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NEURAL CONTROL OF THE PITUITARY GLAND

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THE SECRETORY ACTIVITY of most endocrine glands appears to be outside the direct control of the nervous system. Recent work casts doubt on any nerve supply to the adrenal cortex (274, 392, 396) though experimentally it may be stimulated by the secretion of the adrenal medulla (419). This latter undoubtedly receives a rich innervation from the sympathetic system via the splanchnic nerves and lumbar sympathetic chain (392, 444) and possibly some fibers from the vagus (395). The innervation of the gonads by (in man the tenth thoracic segment) the spinal cord through the spermatic and ovarian plexuses is well known, though the exact site of nerve fiber termination in the gonads is open to dispute (257, 258, 320). However, experimental evidence clearly indicates gonadal activity to be controlled hormonally rather than by a direct nerve supply. The thyroid gland receives nerve fibers from the cervical sympathetic system and vagus (313, 357). Nerve nets around individual follicular cells have been described (391) and their presence denied (314), but the evidence that thyroid activity is under the control of secreto-motor fibers is doubtful. Removal of or stimulation of the cervical sympathetic system (45, 148, 188, 272) may possibly affect thyroid secretion indirectly by a vasomotor influence over the thyroid (357) or pituitary (406). The vagal innervation of the parathyroids (214) appears to be inessential for normal function as demonstrated by transplantation experiments (264). Similarly the pancreas, which receives vagal and sympathetic fibers (351), can still regulate the blood sugar level after vagal section or after transplantation in a depancreatized dog (227). Regarding the nerve supply and control of the pituitary gland, see below.

Thus, of the endocrine glands only the adrenal medulla and neurohypophysis possess a rich nerve supply and are directly influenced in their activity through this supply. (It is of interest to note that developmentally, the neurohypophysis may be classified as the central nervous system and the cells of the adrenal medulla as post-ganglionic sympathetic neurones.) The more slowly acting endocrines are apparently regulated by humoral means. Some activities of the adenohypophysis are clearly influenced by the nervous system, and it may be that nervous control of the endocrine system as a whole is enacted through the mediation of this gland. This surmise would place the hypothalamico-adenohypophysial unit as a key link in the

chain of neural endocrine regulation, and would clarify the position regarding the apparent lack of secreto-motor nerves to the other endocrine organs.

This review is concerned with the evidence relating to neural excitation and control of both the neurohypophysis and adenohypophysis. The terminology used for the various subdivisions of the gland will be that proposed by Rioch, Wislocki and O'Leary (354). The term 'adenohypophysis' (or glandular lobe) includes the pars distalis, the pars tuberalis and the pars intermedia. The neurohypophysis includes the infundibular process (neural lobe) and the infundibulum (neural stalk). The infundibulum may in turn be subdivided into the infundibular stem and the median eminence of the tuber cinereum. The term 'posterior lobe' will be retained to refer to the infundibular process and pars intermedia, and 'hypophysial or pituitary stalk' to include the neural stalk together with its sheath of portions of the glandular lobe.

NEUROHYPOPHYSIS

1. *Nerve Supply of Neurohypophysis*

It is now recognized that the neurohypophysis consists of three parts which function as a unit: the median eminence of the tuber cinereum, the infundibular stem and the infundibular process. That the median eminence is part of the neurohypophysis is shown by its reactions on vital staining (442), vascular supply (171, 437, 438), embryology (401) and cytology (164, 422). Tilney (401) believes the pars tuberalis to be coextensive with the median eminence, but this is denied by Weaver and Bucy (422), who state that the upper boundary of the median eminence is as yet inexactly known. However, the fact remains that the infundibular stem and the lower part of the tuber cinereum probably function as part of the pituitary gland rather than the hypothalamus. Fisher, Ingram and Ranson (140) showed that the median eminence and infundibular stem atrophy (as does the infundibular process) after lesions in the supraoptico-hypophysial tract, and that after simple removal of the infundibular lobe, the presence of the infundibular stem and median eminence are sufficient to prevent the onset of diabetes insipidus. Magoun, Fisher and Ranson (277) found that the median eminence and upper part of the infundibular stem of monkeys constituted 12 to 16 per cent of neurohypophysial tissue and were sufficient to preserve a normal urine output.

The nerve supply of the neurohypophysis is derived from two sources, a scanty sympathetic supply from the carotid plexus running with the posterior median hypophysial artery into the posterior pole of the gland (98) and a hypothalamic supply which reaches the infundibular lobe through the neural stalk. Regarding the sympathetic supply little is known as to the cells of origin, termination or function, whereas the hypothalamic supply, which would appear to be of greater functional significance, has been the subject of much anatomical and physiological study. A comprehensive list of references to work dealing with the anatomy of the hypothalamico-hypophysial tract in all types of vertebrates may be found in the monograph by Fisher, Ingram and Ranson (140).

The hypothalamico-hypophysial tract has been subdivided into the supraoptico-hypophysial tract running in the ventral or more rostral wall of the neural stalk and the tubero-hypophysial tract in the dorsal or more caudal wall of this structure (138, 358).

(*a*) NUCLEI OF ORIGIN OF THE HYPOTHALAMICO-HYPOPHYSIAL TRACT. Ramon y Cajal, 1894 (63) described a tract of fibers running from a nucleus situated behind the optic chiasma into the pituitary stalk and 'superior' lobe in young mice. Pines (335) placed the source of some of these fibers as the supraoptic nucleus (nucleus hypophyseus of Pines). This was confirmed by Greving (176) who described also a tractus paraventricularis-cinereus, arising in the paraventricular nucleus and running either to the supraoptic nucleus or to join the fibers in the supraoptico-hypophysial tract. Laruelle (259) described two groups of paraventriculo-hypophysial fibers, an external and internal set. The similar connection of the supraoptic and paraventricular nuclei with the hypophysis is not surprising in view of their common phylogenetic origin (33) and similar vascular and cytological architecture. In the rabbit some fibers entering the ventral wall of the stalk are said to arise from a group of cells situated behind the chiasma in the midline, termed by Young (445) the nucleus hypothalamicus ventralis pars centralis. Fisher *et al.* (138, 139) describe the origins of fibers in the supraoptico-hypophysial tract as the supraoptic nucleus mainly but also the paraventricular nucleus (filiform), anterior hypothalamic, ventromedial hypothalamic and ventral periventricular hypothalamic nuclei. The tubero-hypophysial tract is said by these authors to arise in the central and posterior parts of the hypothalamus and possibly from the nucleus periventricularis ventralis (343). Scattered cells and nuclei in the tuberal region are also suggested as giving origin to fibers in the lateral and posterior regions of the stalk (358), and there is some evidence that fibers from the pars mammillaris of the hypothalamus enter the tubero-hypophysial component (415).

More recent work on the origin of the hypothalamico-hypophysial fibers has been concerned with the retrograde degeneration that occurs in some hypothalamic nuclei following lesions in the tuber cinereum and infundibular stem. Such lesions are reported to cause clear-cut degeneration in only the supraoptic and paraventricular nuclei, but it is possible that less well-defined nuclei associated with the tubero-hypophysial tract also undergo at least partial degeneration. Kary (248), and Lewy (265) first described retrograde degeneration in the supraoptic nuclei. The normal number of cells in each supraoptic nucleus has been estimated at about 60,000 (man, 305, 346), 30,000 to 40,000 (monkey, 278), 35,000 to 40,000 (dog, 305, 316, 334, 345) and 7,000 (rat, 305, 345). After lesions in the neurohypophysis these cells are considerably reduced in number, the figures recorded varying from 70 per cent loss of cells on removal of the infundibular process to 90 per cent (or more) loss on section of the supraoptico-hypophysial tract above the median eminence (190, 211, 278, 316, 334, 345, 346). Less information is available for the paraventricular nuclei. Many workers have described an apparent loss of cells in this nucleus after partial or complete denervation of the neurohypophysis [Rasmussen (345) rat; Fisher, Ingram and Ranson (139) cat; Biggart and Alexander (21) and Heinbecker and White (211) dog; Rasmussen and Gardner (346) man]. Estimates have been given that about 20 per cent of the cells in the paraventricular nuclei degenerate after pituitary stalk section in the monkey (278), about 90 per cent degenerate after sectioning the supraoptico-hypophysial tract in the dog (316, 334) and 35 per cent degenerate after hypophysectomy in the rat (154). The difference in these estimates is probably due, in part, to the indefinite demarcation of the nucleus. The loss of paraventricular

cells would appear to be greater after denervation or destruction of the median eminence than after pituitary stalk section (211, 316). The loss of supraoptic and paraventricular cells after different lesions is not always proportional, and it has been suggested that some other factor such as interference with the vascular supply of the nuclei may account for this fact (154). O'Connor (316) found that the number of cells remaining in the supraoptic nuclei after various lesions in the supraoptico-hypophysial tract could not be related to the magnitude of the final polyuria.

(*b*) FIBERS IN, AND COURSE OF, THE HYPOTHALAMICO-HYPOPHYSIAL TRACT. The fibers of the hypothalamico-hypophysial tract are usually described as being unmyelinated. Vidal (415) describes these fibers in the rat as losing their myelin sheath at the median eminence. Trocello (402) saw myelinated fibers in the stalk and neural lobe in various forms, and Vasquez-Lopez (412) states that some fibers acquire a myelin sheath as they run to supply what he terms the 'meningeal terminal corpuscles'. Some authors ascribe sensory functions to these fibers (84, 412). It is felt that both the presence of myelinated fibers and the sensory nature suggested for them need confirmation.

As the supraoptico-hypophysial tract enters the median eminence it occupies a very superficial position where it is liable to be affected by basal infections of the brain (139). The fibers here have been described as pursuing a sinuous course (412) and of undergoing partial decussation (259, 359, 360). This decussation is probably not of any magnitude, for unilateral injury of the median eminence (211), or neural stalk (240), causes appreciable retrograde degeneration only in the supraoptic nucleus of the same side. The tubero-hypophysial tract in the dorso-caudal region of the tuber cinereum and infundibular stem is smaller and consists of finer fibers (139). In the neural stalk the fibers are collected into densely aggregated bundles lying in a central position, leaving a peripheral zone in contact with the pars tuberalis, relatively free of nerve elements (412). In the neural lobe the compact arrangement of the nerve fibers is broken up, although two main hypophysial fasciculi have been described in the rat (403). The general picture of fibers in this lobe is that of a tangled, gnarled mesh. Actual counts of nerve fibers in the neural stalk have given figures of about 50,000 for man (344). This is probably an underestimate, for the total number of cells in the two supraoptic nuclei alone exceeds this figure. A closer approximation would probably be 10,000 in the rat, about 60,000 in the dog and monkey and 100,000 in man (345).

(*c*) TERMINATION OF THE HYPOTHALAMICO-HYPOPHYSIAL TRACT. The fibers of the hypothalamico-hypophysial tract end mainly in the neurohypophysis, a few penetrating the pars tuberalis, pars intermedia and possibly the pars distalis. (The fibers that may enter the adeno-hypophysis are discussed below.) Although it is now generally held that the potent extracts obtained from the neuro-hypophysis are formed in situ and not by the pars intermedia (105, 140, 159, 160, 321, 322), it is doubtful which are the 'secretory' elements. The evidence of Gersh (164) that his 'parenchymatous' glandular cells are secretory has been questioned by Hickey, Hare and Hare (218), and it would seem impossible as yet to describe a secreto-motor ending in the neurohypophysis. Histologically the mode of termination of the nerve fibers in the neural lobe is obscure. The lack of a specific stain for nerve fibers (184) and the heterogenous group of structures among which the nerve endings are situated

make it hardly surprising that the fiber terminals are far from clear. [The structures comprising the neurohypophysis may be enumerated as follows, see (343): nerve fibers; nerve cells (377); neuroglia, at least in the form of microglia (411); various cells such as pituicytes (56, 178, 179, 180), mast-like cells (169), wandering cells (57), ependymal lining cells in some forms such as the cat, and cellular cords invading posteriorly from the pars intermedia; a rich vascular supply (442); and the connective tissue framework (56).]

From the evidence given above, that more cells in the supraoptic and paraventricular nuclei degenerate following lesions above or in the median eminence than in the hypophysial stalk, it seems probable that the hypothalamico-hypophysial tract ends in all three parts of the neurohypophysis. Cajal (64) described nerve fibers ending near the surface of the stalk in varicose extremities. Perivascular terminations to the fibers have been described by many authors (89, 184, 397, 412), Tello (397) and Vasquez-Lopez (412) both claiming that this type of ending is particularly numerous and dense in the infundibular stem as compared with the infundibular process. (This point will be referred to again when considering possible modes of transmission of stimuli to the pars distalis.) Other methods of termination appear to be by means of terminal enlargements, bulbs, menisci and similar structures (56, 184, 397) in relationship to cells in the neural lobe (53, 56), in the connective tissue capsule (56), in end organs described as sensory (84, 412) and in the neurohypophysial ependyma (85).

2. *Neural Control of Neurohypophysis*

During the last 50 years various effects produced by administration of extracts of the neurohypophysis have been investigated, the original studies of Oliver and Schafer (323), Howell (231) on the pressor activity, Magnus and Schafer (275), Schafer and Herring (366), Von den Velden (413) on the diuretic-antidiuretic and chloruretic activity, Dale (96) on the oxytocic activity, Borchardt (34) on the hyperglycaemic activity of such extracts, leading the way to the accumulation of much valuable data. Posterior pituitary extracts are now known to influence water and salt metabolism, the cardiovascular system, carbohydrate metabolism, the smooth muscle of the uterus, intestine and other viscera, water balance of amphibians and possibly blood coagulation, fat metabolism and other functions (409). Only recently, however, has evidence accumulated as to the physiological rôle of the neurohypophysis, and it is still not possible to accredit with certainty more than one hormonal function to the organ. Much of the evidence has been derived from studies of experimental or clinical lesions of the organ or its nerve supply. Direct stimulation of the gland has been a less productive field of enquiry owing to the complications associated with anesthesia. Indirect stimulation by means of emotional reactions, peripheral sensory stimuli and other means has yielded valuable information regarding the liberation of the antidiuretic hormone.

The more probable endocrine actions of the neurohypophysis will now be considered with special reference to their loss on damage to, or production on stimulation of, the gland or its nerve supply. Stimulation of the gland will, for convenience, be described as indirect or direct.

(*a*) NEUROHYPOPHYSIS AND THE ANTIDIURETIC HORMONE. The recognition that

the antidiuretic activity shown by extracts of the neurohypophysis represents a true hormonal action is due particularly to the work of Ranson and Verney and their respective colleagues. A detailed account of this work may be found in reviews by Pickford (333), and O'Connor (317).

Lesions. Ranson and his co-workers have demonstrated very clearly the effects of damage to the neurohypophysis or its nerve supply. The disturbance to the water balance of the body (139, 240, 241, 277) (and restoration of normal balance by posterior pituitary extracts), the degenerative changes which occur in the neurohypophysis and associated hypothalamic nuclei (138, 240, 277, 278) and the hormonal content of the atrophic neurohypophysis (136, 137) and other points have been investigated following experimental trauma to the hypothalamus or pituitary. Their work has been made the subject of a monograph (140). The main conclusions of this school are as follows: The supraoptico-hypophysial tract regulates the secretion of the antidiuretic hormone from the neurohypophysis, the term neurohypophysis being taken to include the median eminence, infundibular stem and infundibular process. Under normal conditions in the intact animal the activity of the antidiuretic hormone is balanced against that of the diuretic processes of the body, which appear to be influenced by the adenohypophysis. If the supraoptico-hypophysial tract is interrupted above the level of the median eminence, secretory activity ceases, degeneration and atrophy of the neurohypophysis and major parts of the supraoptic nuclei occur, and a state of diabetes insipidus supervenes. Extracts of the atrophic neurohypophysis show loss of antidiuretic, oxytocic and pressor activity, though the melanophore activity is retained. Major dissentients from these views have little sound evidence in their support (307, 308, 309, 310, 412). Some points still require clarification, such as the part played by the adenohypophysis in the development of a maximum diabetes insipidus (211), and the possibility of extra-hypophysial formation of an antidiuretic principle (251, 301, 302). Keller (251) believes the hypothalamic formation of an antidiuretic substance is a possible explanation of his results, in which complete separation of the hypophysis from the hypothalamus did not give rise to diabetes insipidus. It is generally accepted now that denervation of the neurohypophysis leads to atrophy of the gland and cessation of secretory activity. In this respect, Gellhorn (161) believes the neurohypophysis to be unique among the endocrine organs.

Indirect stimulation. Indirect stimulation of the neurohypophysis either by reflex nervous pathways or humorally has been the explanation put forward to account for a change in rate of urine flow on the application of many different types of stimuli.

Muscular exercise and emotion. For many years it has been known that muscular exercise causes inhibition of a water diuresis. Rydin and Verney (362) produced evidence that the primary inhibiting factor is the emotional accompaniment of exercise. They found that denervation of the kidney (see also 254, 255, 400) did not alter the response, and after excluding the participation of hemodynamic factors suggested that the antidiuresis is due to some agent humorally conducted to the kidney. Further experiments showed that the adrenal glands were not concerned, that the responses to emotional stress and injection of posterior pituitary extracts were closely similar as regards both the urine flow and chloride and nitrogen excretion by the kidney and that removal of the posterior pituitary (318) reduces the inhibition

to about 5 per cent of its original magnitude. O'Connor (315) has also shown that section of the supraoptico-hypophysial tracts in dogs, besides producing the changes described in cats and monkeys by Fisher, Ingram and Ranson (140), very largely abolished the inhibition of a water diuresis produced by emotional stress. Much of the above work was performed on dogs in which the kidneys and adrenals had been denervated. In further work (319) in which this denervation had not been performed, it was found that many dogs respond to emotional stress by a sharp, short inhibition of a water diuresis. After section of the splanchnic nerves, removal of the second, third and fourth lumbar sympathetic ganglia and denervation of the kidneys, a long slow inhibition of pituitary origin was apparent in all cases. This latter could be abolished, however, by injection of adrenaline just before the application of the emotional stimulus, but since similar injections of adrenaline did not abolish the inhibition produced by injection of posterior pituitary extracts, it was concluded that adrenaline acted by preventing the release of the antidiuretic hormone from the pituitary. Tyramine in equipressor doses is as effective as adrenaline in this respect, so it would seem that an increased cerebral blood flow is the common factor in preventing the release of antidiuretic substance (414). This beautiful sequence of experiments shows very clearly that the neurohypophysis may be aroused to secretion by emotional stress, which is a component of the psychological pattern produced by pain, exercise and other natural stimuli. The neural pathway by which this excitation occurs is almost certainly the supraoptico-hypophysial tract. [It is possible that emotional factors may have been responsible for the changes in urine flow described in man (5, 355).]

Hypertonic solutions. The presence of osmoreceptors in nervous connection with the pituitary was postulated by Klisiecki *et al.* (254). The discovery that administration of hypertonic saline inhibits a water diuresis (73, 165, 194, 414) appears to support this view. Chambers reports that the neurohypophysis of the rat, following prolonged administration of hypertonic saline, shows histological changes (71) and a loss of assayable antidiuretic hormone (72). Verney (414) found that the intracarotid injection of hypertonic saline was followed by a definite antidiuresis, which was largely abolished by removal of the posterior lobe of the pituitary. The intravenous injection of the same amount of hypertonic saline had no effect on a water diuresis. It was also shown that the effect is not due specifically to sodium chloride but to the rise in osmotic pressure of the blood, that the osmoreceptors are freely permeable to urea, less freely permeable to dextrose and relatively impermeable to sodium chloride. It seems likely that the antidiuresis evoked by hypertonic saline is in the nature of a physiological response, especially since it has been calculated that the change in blood chloride necessary to elicit the response is well within the range of the falls reported in water diuresis in man (414). The nature and identity of the osmoreceptors is uncertain. The supraoptic nuclei have been suggested as the effective site, largely on the grounds of their highly vascular nature. Also Hare (192) has shown that the antidiuretic response to injection of hypertonic saline is abolished by cutting the pituitary stalk, but if the diencephalon is isolated from peripheral receptors by cutting the midbrain, the cervical sympathetic trunks and the first three cranial nerves, the response is still retained.

Cranial and sensory nerve stimulation. Stimulation of the rat by flashes of light

has been held responsible for indirect stimulation of the neurohypophysis, with a resultant diuresis (36). Similar results had been obtained in the frog which responds with a loss of body water (37). Electrical stimulation of the vagus nerve in the isolated head of the anesthetised dog produced a diuresis and the appearance of an antidiuretic substance in the urine. Hypophysectomy abolished this response, which was attributed to reflex secretion of the antidiuretic hormone (75). Sensory stimulation of the lumbar area has been shown to inhibit a water diuresis (398), the inhibition remaining after renal denervation (400) but not after cauterization of the pituitary stalk (204). Hare (191) however states that inhibition of a water diuresis still occurs after hypophysectomy or pituitary stalk lesions in response to restraint or painful stimuli. Under these circumstances renal clearance tests show the antidiuresis to be due mainly to reduction in the glomerular filtration rate.

Administration of various drugs. Injection of morphine (103), barbiturates (104), nicotine (58), yohimbine (155), atropine (386) or acetyl-choline (332) has been shown to inhibit a water diuresis, and there is evidence that this effect is produced by mediation of the neurohypophysis.

Direct Stimulation. The demonstration that stimulation of the supraoptico-hypophysial tract causes liberation of the antidiuretic hormone is rendered difficult by the fact that administration of anesthetic inhibits a water diuresis. Haterius (204) found that of several anesthetics investigated, a chloralose-urethane mixture injected intravenously had least depressant action on a water diuresis. Under this narcosis, electrodes were inserted into the region of the pituitary stalk of rabbits by means of a stereotaxic instrument in which the animals' heads were clamped and orientated. This manipulation invariably caused abrupt diminution in urine flow, and subsequent gavage frequently resulted in death. However, good diureses were eventually obtained in eight experimental and six control animals. Of the eight experimental animals, five showed a definite antidiuresis on stimulation of the pituitary stalk and three only a transient depression. In one of these three the electrodes were found at post-mortem to be 1 to 2 mm. lateral to the stalk. In the six control rabbits that had had electrolytic lesions placed in the region of the stalk two to four days previously, four failed to show an antidiuresis on stimulation. In the two animals in which stimulation checked the diuresis, autopsy revealed intact pituitary stalks.

In a recent communication (199) electrical stimulation of the neurohypophysis and hypothalamus has been investigated with regard to the secretion of the antidiuretic hormone in the conscious rabbit. Unipolar stimulation was applied by the remote control method, a small coil being buried between the scalp and skull, and an insulated electrode carried down through the skull, corpus callosum, hippocampal commissure and other median structures into some part of the hypothalamus or hypophysis. Rabbits have been shown to live normal healthy lives for periods of at least three years with this unit in situ. After full recovery from the initial operation and anesthetic, stimulation may be performed by placing the animal's head in an electromagnetic field. Repeated experiments may thus be performed on the same animal. With the electrode in, or in contact with some part of the supraoptico-hypophysial tract, short or long-lived antidiuresis could be produced at will, in the

conscious animal, by varying the intensity of stimulation These effects were constant, easily repeatable, and in every way similar to the antidiureses produced by injection of appropriate doses of posterior pituitary extract. Control experiments showed that so far as the nerve fibers in the neurohypophysis were concerned, the effective current spread was ½ mm. or less, indicating a very localized stimulus. If during a water diuresis a given stimulus was repeated the antidiuretic responses tended to increase. The explanation of this phenomenon was not clear for it could not be correlated entirely with a diminishing water load. A given stimulus repeated under identical conditions from day to day gave remarkably constant responses. Sedative doses of a chloralose-urethane mixture tended to depress the response. The antidiuresis following stimulation was unchanged by removal of the left adrenal gland and denervation of the right. A chloruresis was found to occur concurrently with the inhibition of urine flow, the intensity and duration of the two effects running parallel. The chloride concentrating reaction could also be duplicated exactly by intravenous injection of posterior lobe extracts.

(*b*) NEUROHYPOPHYSIS AND THE UTERUS. *Lesions.* Since Dale (96) first reported the oxytocic action of posterior pituitary extracts, many workers have found that normal labor may occur after hypophysectomy or lesions of the neurohypophysis (121, 252, dog; 4, 299, cat; 328, guinea-pig; 298, ferret; 135, rabbit; 371, 384, rat; 372, mouse), and the idea became prevalent that the oxytocic factor was of pharmocological interest only. Some workers suggested the foetal pituitary may play an important part during parturition (15).

Fisher, Magoun and Ranson (141) noticed that pregnant cats which had developed diabetes insipidus following interruption of the supraoptico-hypophysial tract exhibited disturbances in parturition. Of seven animals, four died during labor, two survived for some days and only one remained alive and in good condition. The dystocia took the form either of total inability to deliver the young or a very prolonged labor. The conclusion was drawn that the supraoptico-hypophysial tract and neurohypophysis probably form an integral part of the labor mechanism, and previous work showing normal labor may occur after hypophysectomy was criticized on the grounds that most workers had failed to control the following points: *a*) complete removal of the neurohypopnysis (including median eminence) demonstrated by serial sections after death, *b*) the presence of at least some secretory tissue of the glandular lobe, and *c*) the duration and normality of the various stages of labor. The statement that delivery occurred at term was held to be insufficient. In a further report Dey, Fisher and Ranson (117) confirmed their previous findings. They state that with hypothalamic lesions in the supraoptico-hypophysial tract slightly less than one third of their pregnant guinea-pigs were able to deliver their young normally, and that about one half of them had a definitely prolonged labor or delivered their young dead. Confirmatory results to the above are given by Pencharz and Long (327) from a study of hypophysectomized rats and by Smith (385) for hypophysectomized monkeys. Smith found that of 13 hypophysectomized pregnant rhesus monkeys, 6 completed pregnancy and had normal babies although labor was prolonged, caesarean operations being performed on 3. From this evidence then, it would seem likely that the maternal neurohypophysis is involved in

the process of parturition, but the fact that some animals with diabetes insipidus may deliver their young normally (guinea-pig, 117; dog, 252; and man, 99) makes the evidence not wholly convincing. Further work on the effect of hypothalamic lesions on parturition in different species is needed.

Stimulation. It is claimed (77) that stimulation of the central end of the vagus in the isolated head preparation of the dog causes reflex liberation of the oxytocic factor. This was found to have no effect on the dog's own uterus, but could be detected in the jugular blood by its action on a guinea-pig uterus. Control sera and sera obtained after stimulation in the hypophysectomized dog had no effect on a guinea-pig uterus. Stimulation of the cervical sympathetic in rats has been reported as increasing the amount of oxytocic factor in the pituitary gland (325).

Haterius and Ferguson (206) were the first to investigate the effects of faradic stimulation of the infundibular stem on uterine motility in anesthetized rabbits. They found the response in virgin rabbits to be variable. In the postpartum animal, stimulation produced an increase in uterine activity as shown by increased frequency and amplitude of contractions. This response was simulated closely by an intravenous injection of 'Pitocin,' and from a comparison of the responses they inferred stimulation could cause the liberation of as much as 0.5 U of oxytocic substance at one time. Spinal transection, section of the splanchnic nerves or vagotomy did not abolish the increased uterine motility on stimulation, though an electrolytic lesion placed in the pituitary stalk rendered subsequent stimulation of the structure ineffective. In a further study by Ferguson (133) on postpartum cats and rabbits, it was shown that in animals in which the neck had been crushed (except the carotid arteries, jugular veins and a flap of skin), stimulation of the infundibular stem still evoked an increase in uterine activity equivalent to the injection of 0.5 U 'Pitocin'. Various observations were adduced showing that the increased activity of the uterus was not dependent on an associated rise in blood pressure. In one experiment it was noted that the uterine response to stalk stimulation was more closely simulated by an injection of 'Pitocin' than 'Pituitrin', and the suggestion was made that on stimulation the pituitary liberates a hormone with relatively little pressor activity.

The effects of stimulation of various parts of the hypophysis and hypothalamus have been studied (199) in the unanesthetized rabbit, using the remote-control method of stimulation and a modification of Reynold's (349, 350) uterine fistula for recording uterine activity. In animals retaining their ovaries, uterine activity shortly after operation was usually that of the oestrous state and a marked increase in activity would occur in response to stimulation of the neurohypophysis. These reactions tended to disappear in a few days, possibly because the stimulation also caused a release of luteinizing hormone from the adenohypophysis. Good standard preparations were obtained, however, following ovariectomy and the subcutaneous implantation of a tablet of stilboestrol di-n-butyrate. Stimulation of the median eminence or infundibular stem in the oestrous or oestrogenized rabbit was shown to cause a very marked increase in uterine activity. The response to a given stimulus in the oestrogenized rabbits was very constant from day to day. The magnitude of the effect could be graded at will by varying the intensity of the stimulus and could be duplicated exactly by the intravenous injection of posterior lobe extracts in doses up to 200–500 mU. Similar stimuli applied in control rabbits

with the electrode tip in the pars distalis below the infundibular stem, in the posterior part of the tuber cinereum above the infundibular stem, or in the region of the paraventricular nucleus were ineffective in altering uterine motility providing stimuli were applied at a distance greater than ½ mm. from the supraoptico-hypophysial tract. A further group of control animals in which stimuli were applied not directly to, but within ½ mm. of the ventral, lateral or dorsal sides of the infundibular stem showed submaximal increases in uterine activity. (The magnitude of the antidiuretic responses in these same animals could be correlated in a similar manner with the site of stimulation.) The uterine responses to stimulation of the neurohypophysis and to intravenous injection of posterior lobe extract were also observed through periods of anoestrus, pseudo-pregnancy and progesterone treatment and found to be qualitatively similar. Deep ether anesthesia inhibited the uterine response to stimulation markedly, but had much less effect on the response to injection of posterior lobe extract; light anesthesia (intravenous chloralose-urethane) had little inhibitory effect on the uterine reactions. Removal of the left adrenal gland and denervation of the right did not alter the uterine response to stimulation of the median eminence. Administration of adrenaline had an effect on the uterus qualitatively dissimilar to that following stimulation of the neurohypophysis or injection of posterior lobe extracts.

The data given above indicate that, in the rabbit and cat at least, the neurohypophysis has the potentiality of secreting an oxytocic factor into the blood stream which can exert a marked effect on the uterine musculature. The proof that this oxytocic principle is a true hormone rests on the demonstration that such a release and action occur in response to 'natural' stimuli. It has been suggested (199) that the act of coitus, known to stimulate the adenohypophysis of the rabbit, stimulates also the neurohypophysis, and thus may increase uterine motility and aid sperm transport. The fact that stimulation of the vulva of the rabbit increases uterine motility (207) and the rate of transmission of fluids up the uterus (256) could be adduced as evidence in favor of this view. Ferguson (133) has described a reflex release of oxytocic substance from the neurohypophysis following the stimulus of distension applied to various parts of the birth canal and has suggested that reflexes of this type may play a part in the mechanism of parturition.

(*c*) NEUROHYPOPHYSIS AND BLOOD SUGAR. Borchardt (34) first showed that injection of extracts of the posterior lobe of the pituitary produces a hyperglycemia and glycosuria. This observation has been confirmed and extended, the general conclusions (409) being that posterior lobe extracts on injection will produce a moderate hyperglycemia persisting for a few hours and often followed by a fall in blood sugar. Most work indicates the vasopressor principle to be the active constituent of such extracts, although some (130, 225, 230) believe the oxytocic principle to be more potent in this respect. The doses of extract necessary to produce hyperglycemia are large. In the rabbit the following doses have been used: 2–4 U/kgm. body weight, intravenous injection of 'Post-Lobin V' (129); 7½ U posterior lobe extract injected intravenously (66); 20–60 U 'Pituitrin' injected subcutaneously (436). Since the pituitary of the rabbit has been estimated as containing only 1.3–2.5 U of the pressor principle (381), these doses appear to be outside the physiological range.

Injuries to the hypothalamus have been stated by different authors to cause

both hyperglycemia or hypoglycemia. If these changes in blood sugar are mediated via the pituitary gland, it would seem likely that they are related to the activity of the adenohypophysis rather than the neurohypophysis and will be discussed further below. Keller (250), however, suggests that the decreased tolerance to insulin found in cats with large hypothalamic lesions is due to lack of secretion by the neurohypophysis.

Electrical stimulation of the cervical sympathetic system, or hypophysis itself, has been described by many workers as causing a glycosuria or hyperglycemia. Weed, Cushing and Jacobson (423) claimed stimulation of the hypophysis produced a glycosuria in cats and dogs, even after preliminary transection of the spinal cord and cervical sympathetic trunk, and that stimulation of the superior cervical ganglion in cats, rabbits or dogs by faradization or by the manipulation necessary for its exposure likewise resulted in a glycosuria. This latter reaction remained after section of the cervical sympathetic trunk, spinal cord and vagi but was abolished by removal of the posterior lobe of the pituitary. They suggested the infundibular process is innervated by the superior cervical ganglion, and that stimulation of any part of this mechanism liberates posterior lobe hormone so producing a glycogenolysis. Direct stimulation of the pituitary gland has been described by Keeton and Becht (249) as causing a glycosuria, abolished by previous section of the splanchnic nerves, and by Ingram and Barris (238) as causing a glycosuria often accompanied by a diuresis. Harris (199) found that stimulation of various regions of both the neurohypophysis and adenohypophysis of the unanesthetized rabbit by the remote-control method failed to produce a hyperglycemia or diuresis that could be ascribed to pituitary activation, and suggested that the results of previous workers had been complicated by the administration of an anesthetic. That stimulation of the cervical sympathetic system causes a glycosuria or hyperglycemia has been confirmed (100, 233, 376) and denied (219, 342). These experiments were all performed under anesthesia, except those of Rabens and Lifschitz (342) who criticize the results of previous workers as being due to prolonged anesthesia. Stimulation of the central end of the cut vagus nerve has also been reported to cause hyperglycemia due to reflex excitation of the neurohypophysis (233).

There is little evidence that the neurohypophysis plays a physiological rôle in the regulation of blood sugar.

(*d*) NEUROHYPOPHYSIS AND BLOOD PRESSURE. In 1895 Oliver and Schafer (323) reported that injection of pituitary extracts into anesthetized animals raised the blood pressure. Howell (231) showed the active principle was contained in extracts prepared from the posterior lobe and not from the anterior lobe of the pituitary gland. As is well known, these observations have been amply confirmed, and a transient depression of blood pressure, which often precedes the more prolonged rise following injection, has been ascribed to a constriction of the coronary arteries with impairment of the heart's action and diminution in cardiac output (303 and others). Injection of whole or fractionated posterior lobe extracts in unanesthetized animals or man is commonly stated to lower the systolic blood pressure and cardiac output, effects which are attributed to coronary constriction and reflex nervous activity (181, 182, 183), but pressor responses are also described (198, 367).

Total hypophysectomy results in a fall of blood pressure in the rat (260, 443) and dog (39), but removal of the pars neuralis alone (39, 443) does not produce hypotension. In view of these results it might be expected that lesions in the nerve supply of the neurohypophysis would affect the blood pressure little, if at all. Section of the pituitary stalk in a case of malignant hypertension produced a marked reduction in blood pressure (346), but the significance of this case is doubtful, the result possibly being secondary to vascular damage to the pars distalis. In dogs showing experimental hypertension of the Goldblatt type, lesions in the supraoptico-hypophysial system produced a fall in mean blood pressure (364), and one normal dog in which the median eminence was sectioned showed a fall of blood pressure for one month followed by a return to normal levels, though a marked polyuria was maintained. The evaluation of these results is difficult.

Stimulation of the neurohypophysis by reflex pathways has been held responsible for the pressor response in the trunk produced by stimulation of the central end of the vagus nerve in the isolated head of the dog (74). This rise was abolished by cutting the pituitary stalk or by hypophysectomy and was duplicated by the intravenous injection of 100 mU 'Pitressin' (see also 363). An attempt to trace the nervous pathway involved has been made (234). Similar reactions have also been obtained in the toad, turtle, chicken, duck, rabbit and cat, but not the snakefish (273). Stimulation of other cranial nerves (olfactory, optic, lingual, vestibular, glossopharyngeal and hypoglossal) also produces, in the dog preparation, slight pressor responses abolished by hypophysectomy (235). Repeated stimulation of the central vagus causes exhaustion of the pressor response which returns with rest. These changes have been correlated with the exhaustion and reappearance of granules in pituicytes (76), but in view of the work of Hickey, Hare and Hare (218), the significance of granules is open to doubt. That the arterial hypertension due to stimulation of the central vagus is abolished by hypophysectomy has been denied (293) and partially confirmed (363). It has been suggested that thermal trauma, which elicits a pressor response, may activate the neurohypophysis (324).

Direct electrical stimulation of the pituitary gland or its hypothalamic nerve supply has been investigated with regard to a possible pressor response. Cyon (93, 94, 95) one of the first workers to stimulate the pituitary gland directly reported a bradycardia and rise in blood pressure as resulting from mechanical and electrical stimulation of the gland. Stimulation of the supraoptic region of the anterior hypothalamus of the anesthetized spinal cat was reported by Clark and Wang (79) to give a pressor response. The characters of this response were a long latent period, a slow smooth rise of about 30 mm. Hg, and a duration of approximately two to four minutes, indicating the possibility that the response was activated humorally through the supraoptico-hypophysial tract and the neurohypophysis. These results have been confirmed, striking elevations in blood pressure being obtained on bipolar stimulation of the infundibulum in animals with crushed spinal cords (Sattler, 363). Ferguson (133) records small rises in blood pressure on stimulating the pituitary stalk in rabbits and cats anesthetized with chloralose-urethane and in which vascular connections only remained between the head and trunk. In some cases the pressor response was very slight (see his fig. 2), although the oxytocic effect of the stimulus

was well marked. In view of the different effects recorded following injection of pituitary extracts into anesthetized and unanesthetized animals, observations were made (201) on the response of the conscious rabbit to injection of posterior pituitary extract and remote control stimulation of the pituitary stalk, using Grant's capsule (167) for recording the blood pressure. The unanesthetized rabbit exhibits a pressor response on intravenous injection of 'Pituitrin', and on stimulation of the infundibular stem slight pressor responses (less than those evoked by 100 mU of 'Pituitrin' injected intravenously) of a similar type were obtained.

(*e*) NEUROHYPOPHYSIS AND INTESTINAL PERISTALSIS. There are few subjects in the literature on which more discordant results have been recorded than the effects of posterior pituitary extract on intestinal motility, and there would seem little justification at the present time for correlating results obtained by in vitro experiments on isolated intestinal segments, and in vivo experiments performed with and without the use of anesthetics on a variety of animal species and with a wide variation in dosage of extracts used.

There is some evidence available, however, concerning the effects of stimulation of the neurohypophysis or its nerve supply on intestinal motility. Wang, Clark, Dey and Ranson (421) found that electrical stimulation of the region anterior to the infundibulum of the anesthetized cat produced an excitation of gastro-intestinal motility. This response had a long latent period, persisted for several minutes after cessation of the stimulus, was not abolished by sectioning the vagi, but was seen in slight form in only 4 out of 11 cats after sectioning the spinal cord. In view of this last observation the authors were unwilling to emphasize the participation of the neurohypophysis in the reaction. Vagal effects on intestinal motility, observed when the hypothalamus, at or behind the infundibular level, was stimulated, were more immediate in onset and completion. Relative to these results are those of Steggerda, Gianturco and Essex (387), who found the threshold dose to activate the colon of the conscious cat was two to three pressor units injected intravenously and that no response was obtained after injection of 'Pitocin' or 'Pituitrin'. Wang *et al.* (421) found intestinal motility increased in three out of five cats after injection of four pressor units. Assays of the pressor content of the cat's neurohypophysis have been estimated at about 6 pressor units (140) and 5.6–13.1 pressor units (381). It would seem possible then for the gland of this animal to secrete a sufficient amount of pressor factor to activate the intestines, but the evidence that this occurs is incomplete, since Ferguson (133) found the response to stimulation of the pituitary stalk in cats was about equivalent to that obtained by injection of ½ U. 'Pitocin' and that the secretion appeared to have relatively less pressor than oxytocic activity.

In the case of the rabbit, it has been shown that the colon is the most sensitive part of the alimentary canal to the action of posterior lobe extracts, and that the pressor rather than the oxytocic is the active fraction (158, 304). The effect of stimulation of the infundibular stem and median eminence on intestinal peristalsis has been studied in the unanesthetized rabbit (201). It was found that the peristaltic waves as observed through the intact abdominal wall of the unanesthetized rabbit tied in the supine position are small in amplitude and infrequent or, more usually, absent. On intravenous injection of posterior lobe extract (50–500 mU), a

marked increase in the visible waves occurs in the right lumbar and umbilical areas, that is, in the regions overlying the colon and cecum. This increase occurs after a latent period of about thirty seconds and persists for many minutes. On stimulation of the median eminence or infundibular stem a similar increase in peristalsis was observed. This response had a somewhat longer latent period (30–90 seconds), though in other respects it was similar to that following injection of posterior lobe extracts in doses less than 100 mU. In these experiments the stimulus spread, with regard to the supraoptico-hypophysial tract, was less than $\frac{1}{2}$ mm. It is possible then, that colonic activity in the rabbit may be excited humorally by the neurohypophysis, which is in turn under the neural control of the hypothalamus.

3. *Nature of the Substance Liberated from the Neurohypophysis on Electrical Stimulation of the Supraoptico-hypophysial Tract.*

The nature of the secretory product of the neurohypophysis has been discussed for many years. Early views have been summarized (247). The view that the gland secretes more than 1 active principle (multiple hormone theory) has been strongly contested by Abel and his co-workers (1, 2), who support the 'unitary theory' that the gland secretes a single specific substance with multiple actions. Two closely related problems are involved; first the nature of the substance(s) elaborated and stored by the gland and second the nature of the substance(s) liberated from the gland into the blood stream. Van Dyke, *et al.* (410) have isolated a protein from the dried posterior lobe of frozen gland material which behaves as a homogenous substance to solubility, electrophoresis and ultra-centrifugation tests, and which possesses oxytocic, pressor and anti-diuretic activities in the same proportion as those of the U. S. P. reference standard. These authors suggest that even if the pars neuralis elaborates a single protein with multiple activities, it is possible that specific enzymes liberate active fragments of the parent molecule into the blood stream, depending upon the requirements of the organism. As is well known posterior lobe extracts may be fractionated into preparations showing a high degree of separation of pressor and oxytocic activities (242, 247, 388). The pressor principle is usually considered responsible for the renal action of extracts, but according to Heller (212) some separation of these activities is also possible. Fraser (146) maintains that the hydrolysis, as used by Heller, involves no real change in the ratio of antidiuretic and pressor activity.

A few observations indicate that the substance(s) liberated from the neurohypophysis on stimulation of its nerve supply exert relatively less pressor (or antidiuretic) activity as compared with oxytocic, than do whole posterior lobe extracts. Ferguson (133) noted in one experiment that stimulation of the pituitary stalk was more comparable in its proportionate effects on the uterus and blood pressure of the anesthetized animal to one U. 'Pitocin' than one U. 'Pituitrin'. In other experiments he noted the uterine contraction elicited by stimulation of the stalk was qualitatively similar to that produced by injection of 'Pitocin' rather than 'Pituitrin'. He concluded that stimulation of the pituitary stalk liberates a hormone from the neurohypophysis with little pressor activity. Harris (199) has found that stimulation of the median eminence or infundibular stem in rabbits causes a substance to be

liberated from the neurohypophysis which possesses less antidiuretic in proportion to oxytocic activity than various standard unfractionated extracts. Stimulation was performed in the unanesthetized state, the antidiuretic and oxytocic activities being measured simultaneously and compared with similar responses evoked by intravenous injection of posterior lobe extracts into the same animals over a series of days. Also the closer correspondence between the uterine contraction produced by stalk stimulation and injection of 'Pitocin' rather than 'Pituitrin' was confirmed. Injection of 'Pituitrin' caused a greater degree of inhibition of uterine activity following the initial contraction than either of the other two procedures, this probably being due to the higher pressor content producing spasm of the uterine vessels (311). It has also been found (201) that, whereas stimulation of the infundibular stem or median eminence produces an oxytocic action equivalent to about 250 mU. of posterior lobe extract, similar stimulation produces an effect on the blood pressure and intestinal peristalsis less than that evoked by 100 mU. of such extracts. On the assumption that the pressor and antidiuretic activities run parallel, and that the action on intestinal motility is due to the pressor principle, there would seem to be substantial evidence for the belief that the secretion of the neurohypophysis produced by stimulation of the supraoptico-hypophysial tract in the rabbit possesses relatively low pressor activity.

Recently it has been shown (201) that the reactions of the oestrogenized uterus, following intravenous injection of a solution containing 'Pitocin' and 'Pitressin', gives a roughly quantitative assay of the 'Pitocin' : 'Pitressin' content of such mixtures. The 'Pitocin' content is related to the initial uterine contraction and the 'Pitressin' content to the subsequent inhibitory phase. One minute stimulation of the infundibular stem by the remote control method in the rabbit produces a secretion equivalent (in action on the uterus) to a mixture of 250 mU. 'Pitocin' and 50–100 mU. 'Pitressin.' One hour after intravenous administration of hypertonic saline, a similar stimulus produces a secretion equivalent to about the same amount of 'Pitocin' but less 'Pitressin.' After various control procedures the most likely explanation appears to be that the injection of hypertonic saline has depleted the neurohypophysis of its pressor content, but not the oxytocic.

ADENOHYPOPHYSIS

1. *Evidence That the Adenohypophysis Is under Neural Control*

There is much evidence that the adenohypophysis is under neural control but only conflicting evidence regarding the presence of a direct secreto-motor nerve supply to the gland.

The evidence that the nervous system affects the secretory activity of the pars distalis of the hypophysis may be conveniently summarized as follows.

(*a*) INDIRECT EVIDENCE. EXTEROCEPTIVE FACTORS WHICH ACT UPON THE NERVOUS SYSTEM AFFECT ORGANS IN WHICH DIRECT NERVOUS CONTROL IS UNLIKELY, BUT WHICH ARE KNOWN TO BE INFLUENCED BY PITUITARY HORMONES. (i) *Gonads.* The effects of exteroceptive stimuli on the reproductive processes of vertebrates have been the subject of several recent reviews (25, 286, 288, 361 and especially 289). The intrinsic sexual rhythm is in many vertebrates adjusted to external seasonal changes,

so that variation in the seasonal environment produces a corresponding adaptation in the reproductive processes. One example of this phenomenon is the change-over that occurs in the oestrous cycle of some mammals after transference across the equator (287). The active factors of the general environmental pattern are in many cases doubtful. Light has been shown to be a potent stimulus to the sexual processes in some fishes, amphibians, reptiles and many birds and mammals (289). Among the common mammals the reproductive rhythm of the mouse (9, 175), rat (55, 109, 142, 337, 404), ferret (23), hedgehog (3), cotton-tailed rabbit (28), cat (101), raccoon (27), goat (26) and sheep (470) is sensitive to changes in light exposure, while that of the adult cotton rat (306), squirrel (246, 426), guinea-pig (107) and rabbit (382) is not. Other environmental factors which may alter the reproductive processes in mammals and birds are temperature and humidity (10, 32, 261, 287, 427), visual stimuli (295) and tactile or proprioceptive stimuli (393). The relative constancy of the number of eggs in a clutch for any particular species of bird, and the repeated laying of the bird if by removal of eggs from its nest it is prevented from attaining its usual quota (289), again point to an influence of visual or tactile stimuli on the adenohypophysis. Desclin (112) believes that the act of suckling exerts a direct influence on the basophil cells of the maternal pituitary and consequently on its gonadotropic activity.

The presence of a sterile mate or companion of the same sex may exert a marked effect on the sexual rhythm. Exteroceptive stimuli of various kinds would appear to be involved. Olfactory and auditory stimuli probably play a part in some forms (59), though little is known about these. Brooks (49) showed that removal of the olfactory lobes and neocortex or destruction of the labyrinths and cochleae in the female rabbit does not prevent mating and subsequent ovulation, but that ablation of the neocortex and olfactory bulbs in the male rabbit permanently abolishes mating behavior. Tactile or proprioceptive stimuli appear significant in many forms, but especially in those animals which ovulate only after sexual excitement or mating [viz. rabbit (208), ferret (187, 285), cat (102, 271), ground-squirrel (145), short-tailed shrew (326) and mink (189)], though the work of Brooks (49) on the rabbit indicates that the stage of sexual excitement necessary to elicit discharge of the pituitary secretion may be produced by stimuli from a variety of sensory end organs. For a review of the factors underlying sexual behaviour in mammals see Beach (14). The sight of another animal is occasionally sufficient to produce ovulation in the rabbit, and more particularly, in the pigeon. In the latter case even the reflected image from a mirror may produce the same result in the isolated bird (295). The sex display of many birds probably affords another example of this type. Marshall (286, 288) has suggested that the main function of visual stimuli in the form of sex display and courtship phenomena among various classes of animals is to promote an effective synchronization of the male and female sexual processes, thus favoring successful procreation. He suggests this synchronization is mainly effected by pituitary stimulation through the intermediation of the hypothalamus.

(ii) *Breasts, thyroid, adrenals.* The maintenance of milk secretion by the mammary glands is largely dependent on the exteroceptive stimulus of vigorous suckling (373, 374). Since there is evidence that afferent nervous pathways from the nipples are involved (see below) and that the breast lacks a secreto-motor nerve supply, the

most likely hypothesis would appear to involve a reflex stimulus to the secretion of the lactogenic hormone by the pituitary gland.

Similarly the changes which occur in the thyroid and adrenal cortex under the influence of seasonal variations or experimental changes in the environmental temperature (19, 109, 448, 449) may be due to reflex alteration in the activity of the adenohypophysis, since the thyroid gland appears to receive only a vasomotor innervation, and the adrenal cortex lacks any nerve supply. Brolin (46) gives a detailed discussion of the effect of environmental temperature on the thyroid and adrenal glands.

(iii) *Clinical data.* Evidence of a similar nature to that presented above may be adduced for man. It is well known that the usual menstrual rhythm may be interrupted by environmental change [viz., the women of Patagonia and Eskimo women during the long winter nights (268)], by suckling and lactation and by worry or fright (269, 399). The process of lactation is also greatly affected by the emotional state. Cases of exophthalmic goiter (20, 267) frequently present a history of psychic trauma preceding the onset of the disease. A possible mechanism underlying these reactions is a nervous, reflex activation or inhibition of the adenohypophysis resulting in a hormonal effect on the ovaries, breasts or thyroid.

(*b*) DIRECT EVIDENCE. DAMAGE OR STIMULATION OF POSSIBLE NERVE PATHWAYS TO THE ADENOHYPOPHYSIS. The large amount of evidence derived from experimental studies of this type will be discussed below when the individual nervous pathways to the pars distalis are under consideration. Clinical observations, in which lesions of the hypothalamus and other parts of the brain have been found to interfere with sexual development and function, are numerous (see 51). Hypothalamic lesions have been held responsible for hypogonadism, either alone (177) or in association with Frohlich's adiposogenital syndrome (122, 353, 389) or the Laurence–Moon–Biedl syndrome (347). Hecker and Warren (209) concluded that many changes seen in the Laurence–Moon–Biedl and Frohlich's syndromes are due to involvement of the hypothalamus and, indirectly, the pituitary. Pineal tumours may also be associated with genital dystrophy, but it seems likely that this disturbance is due to secondary involvement of the hypothalamus (81, 353). Since hypogonadism may be a non-specific change, due perhaps to a state of malnutrition associated with the primary lesion, better evidence concerning a possible hypothalamico-hypophysial relationship is offered by a study of cases showing precocious puberty in association with neural lesions. That hypothalamic lesions may cause pubertas praecox is well known (47, 120, 122, 144, 341). In these cases the lesion is usually situated at the level of, or caudal to, the tuber cinereum, and it has been suggested (425) that the precocity is due to destruction of a posterior hypothalamic mechanism with release of an anterior hypothalamic innervation of the pars distalis. Weinberger and Grant (425) review the literature on this subject. Changes in thyroid activity associated with a cyst near the infundibulum (312) and other lesions of the brain (267, 336) have also been reported. Three cases of thyrotrophic exophthalmos, possibly of pituitary-diencephalic origin, have been described by Zondek and Ticho (450). In view of the known actions of the diabetogenic hormone the fact that hypothalamic lesions are occasionally found associated with diabetes mellitus (420) may indicate that a similar mecha-

nism underlies these cases, as suggested above to explain pubertas praecox with hypothalamic lesions.

(*c*) EVIDENCE DERIVED FROM THE STUDY OF HYPOPHYSIAL TRANSPLANTS. In studying the replacement capacity of hypophysial grafts in hypophysectomized animals, attention should be paid to the following points. First, the grafted tissue should be situated at a distance from the normal site of the gland thus obviating the danger of vascular or nervous repair; second, the studies should be extended over a period sufficient to ensure that any effects observed are due to functional activity of the transplant, and not to absorption of an implant; and third, serial sections through both the transplant and sella turcica with surrounding tissue are necessary to control the activity of the graft and the complete removal of the pituitary.

The male reproductive organs in hypophysectomized animals are reported to be maintained or restored to function by ocular grafts of the pituitary in rats (296) and (incompletely hypophysectomized) mice (221) by transplants in the sella turcica in rats (91, 174) and by ocular transplants in rats (91) and guinea-pigs (369). Cutuly (91) obtained fertile matings from two male rats 200 and 209 days after hypophysectomy and transplantation, in spite of the fact that the testicular weights of his experimental animals were all below the average.

In hypophysectomized female rats, pituitary transplants have produced inconstant effects on the reproductive organs. Martins (294) found only 1 out of 4 animals exhibited oestrous changes, and these cycles were irregular. May (297) obtained normal oestrous cycles with ocular transplants, but these cycles were not entirely due to the transplants, for removal of the eyeball bearing the grafted tissue did not immediately result in their loss. Greep (174) has reported normal reproductive processes (oestrous cycles, pregnancy, parturition and lactation) in rats bearing transplants in the sella turcica. Loss of oestrous cycles in hypophysectomized rats bearing intramuscular (330) or ocular (92, 431) transplants has been reported more recently. Hypophysectomized female guinea-pigs bearing ocular pituitary transplants show follicular development, hypertrophy of the uterus and proliferated mammary glands, though cyclical activity is lacking and a state of constant oestrus finally intervenes (368). It is perhaps significant that the most complete replacement of pituitary function was that obtained by Greep (174) with grafts in the sella turcica, a position in which regeneration of nervous or vascular connections may possibly occur.

An increase in body weight following transplants in hypophysectomized animals has been reported by several observers (91, 174, 296, 368, 369, 431), though in most cases the increase is slight or less than in controls (91, 174). Maintenance by transplants of the thyroid gland has been observed by Schweizer, Charipper and Haterius (368) though not by Cutuly (91). Differing accounts have been given regarding maintenance of the adrenals (91, 221, 368, 369, 431).

2. *Possible Nerve Pathways to the Adenohypophysis*

From the above data it would appear that there is strong presumptive evidence in favor of some degree of neural control over the adenohypophysis. The mechanism whereby this neural control is exerted is uncertain. The nervous system may affect the secretory activity of the pars distalis either by a direct nerve supply through the

mediation of the cervical sympathetic system, the parasympathetic system via the spheno-palatine ganglion or petrosal nerves, or the hypothalamico-hypophysial tract; indirectly through a neurovascular pathway involving the hypophysial portal vessels; or by some other unknown route. At the present time the balance of evidence is in favor of the view that nervous stimuli affect the pars distalis by means of humoral transmission through the hypophysial portal vessels. This hypothesis will be discussed in detail after consideration of the anatomical and experimental evidence.

(*a*) CERVICAL SYMPATHETIC SYSTEM. The first account of any nerves passing to the pituitary gland was brief mention of a sympathetic supply by Bourgery in 1845 (35). Henle (213) described small nerve twigs passing from the carotid plexus medially to the hypophysis, which, he said, had been previously described by Fontana, Ribes and Bock. Berkley (18) stated that only sympathetic fibers were to be found in the anterior hypophysis of the dog. The first detailed description of the sympathetic pathway to the gland was given by Dandy (98) who used the intravitam methylene blue method of staining. He described the nerve supply of the glandular hypophysis as passing with the arterial supply from the circle of Willis down the stalk to the gland. These nerve fibers came mainly from the plexus around the posterior communicating artery, and as many as three or four small nerve filaments may accompany an arterial branch to the glandular lobe. Pines, (335) using methylene blue and silver staining methods, also demonstrated a sympathetic innervation of the pars glandularis. Hair (184) found a rich innervation of the pars glandularis running with two sets of vessels, first with vessels from the circle of Willis running directly to the pars distalis and second with vessels passing via the pars tuberalis, where an extensive vascular network was found. Rasmussen (344), in a comprehensive study of the nerve fibers to the human pituitary supplemented by observations on the rat, guinea-pig, rabbit, cat, dog and monkey, found sympathetic fibers from the cavernous plexus entering the pars distalis. Large areas of the gland were found to be free of nerve fibers, however, the conclusion being drawn that the fibers present were probably vasomotor in nature. Truscott (403) estimated that out of 2,000 nerve fibers entering the glandular lobe of the rat's pituitary, 20 per cent are carried in on the walls of blood vessels. Drager (123, 124) states that the pars tuberalis of the porpoise and bird receives both autonomic and hypothalamic nerve fibers, but that none pass on to the pars distalis. In the armadillo (125) this author describes sympathetic fibers from the carotid plexus (as prominent nerve fiber bundles) which end in contact with, or between, the glandular cells of the pars distalis.

Maiman (281) found chromatolytic changes in the superior cervical ganglion after destruction of the anterior lobe in dogs. This result was not confirmed by Mahoney (279) in the chimpanzee. Nerve fibers are still present in the pars distalis of the cat (184), rat and rabbit (53) after superior cervical ganglionectomy. In the rabbit they are said to be less than normal in number (53). It should be remembered that sympathetic fibers with cell stations in the scattered ganglia along the internal carotid artery may reach the gland, or alternatively, that fibers from the vertebral plexus may be concerned. Phillips (331) recorded action potentials from the pars distalis during stimulation of the cervical sympathetic trunk. Collin and Hennequin (86, 87, 88) and Popjak (340) have observed histological changes in the pituitary and hypothalamus, respectively, after removal of the superior cervical ganglion.

Many fragmentary claims have been made that stimulation or removal of the cervical sympathetic system affects the secretion of the gonadotropic hormone by the pars glandularis. Several workers have shown (147, 202, 416) that cervical sympathectomy abolishes or reduces the pseudopregnancy response that normally follows artificial stimulation in the rat. (Pseudo pregnancy in the rat, and ovulation with consequent pseudopregnancy in the rabbit, may be induced by sterile mating or some form of artificial stimulation. There is much evidence that in these reactions, the anterior lobe of the pituitary gland is activated by a nervous reflex.) Ball (11) thinks a quantitative variation in the stimulus may be, at least partially, responsible for this result. Friedgood and Bevin (149) also report that removal of the superior cervical ganglion, cervical sympathectomy or blank neck operations may induce an immediate pseudopregnancy, while Britt (41) found psychic stimuli, which previously caused an oestrogenic response in rats, were ineffective after sympathectomy. In a few rabbits, Friedgood and Pincus (152) produced ovulation and maturation of ova by stimulation of the cervical sympathetic trunk, but more recently Friedgood and Cannon (150) failed to obtain ovulation following sympathetic stimulation, though maturation of ova occurred. These results are unconvincing as a demonstration of a secretory nerve supply to the adenohypophysis, for the specificity of the pseudopregnancy response in the rat would appear in doubt, and the slight ovarian changes in the rabbit may have been due to vasomotor effects on the hypophysial vessels. The large mass of negative evidence forces the conclusion that the sympathetic system plays little part in the control of secretion of the gonadotrophic hormones. Pseudopregnancy still follows sterile coitus in the partially sympathectomized rat (147, 202, 416), ovulation still follows sterile coitus in the partially or completely sympathectomized rabbit (48, 203, 224, 417) and cat (69) and may follow stimulation of the tuber cinereum after cervical sympathectomy in the rabbit (196). Stimulation of the cervical sympathetic nerves has failed to produce ovulation in the rabbit in the hands of most workers (150, 193, 203, 284). Perry (329) obtained not a stimulation but a regression of the reproductive system following administration of adrenalin to female rats.

It has been claimed that the cervical sympathetic system may affect the output of the thyrotropic hormone from the adenohypophysis (143, 406). Stimulation of the cervical sympathetic either by anastamosis with the phrenic nerve (151) or electrically (188) has been found to increase the metabolic rate. Contrary results have, however, been reported (45, 148, 283). More significant evidence has been obtained by Brock, Doty, Krasno and Ivy (45), who obtained a marked fall in metabolic rate in seven out of nine rabbits and two out two cats following cervical sympathectomy, and by Uotila (406) who noted a temporary and mild hypoactivity of the thyroid in rats following a similar operation. It should not be forgotten, however, that removal of the sympathetic system in cats (69) and rats (262) may be performed without any very significant change in the metabolic rate, and further that the cervical sympathetic system may well exert a vasomotor control over the hypophysial or thyroid vessels.

(*b*) OTHER AUTONOMIC NERVES. Cobb and Finesinger (83) and Chorobski and Penfield (78) described parasympathetic fibers in the cat and monkey which run from the greater superficial petrosal nerve to join the carotid plexus. These were shown

to carry vasodilator fibers for the pial vessels. Hinsey and Markee (224) suggested this pathway might be involved also in transmitting secretomotor impulses to the adenohypophysis. In the rat, Zacharias (446) reported that a branch from the Vidian ganglion at the junction of the superficial and deep petrosal nerves runs to at least the capsule of the pituitary gland.

Experimental support for a parasympathetic innervation of the adenohypophysis appeared to be forthcoming when it was found that anesthetization of the nasal mucous membrane (378) or extirpation of the spheno-palatine ganglion (356) was followed by pseudopregnancy in approximately 50 per cent of rats. It was further found (447) that removal of the Vidian ganglion produced a pseudopregnancy response in 100 per cent of the cases, and an increased insulin sensitivity in 66 per cent, although section of the greater superficial petrosal nerve which caused pseudopregnancy in a high percentage of cases had no effect on insulin sensitivity. This possible innervation of the adenohypophysis cannot be of more than subsidiary importance, for female rats deprived of the sphenopalatine ganglion show normal sex functions as far as the date of the vaginal opening, reproductive capacity and pseudopregnancy response to stimulation of the uterine cervices are concerned (379). In the rabbit also, bilateral avulsion of the facial nerve and geniculate ganglion (185) and bilateral section of the greater superficial petrosal nerve (418) did not prevent ovulation following coitus. The immediate pseudopregnancy response of rats following operative trauma would appear to be a non-specific reaction, for Friedgood and Bevin (149) report pseudopregnancy occurs in 64 per cent of animals following bilateral removal of the superior cervical ganglion, in 39 per cent following bilateral cervical sympathectomy and in 23 per cent following blank neck operations. The fact that in the rabbit ovulation follows coitus even after the intravenous injection of large doses of atropine (282) again suggests the parasympathetic system plays little part in this response.

(*c*) HYPOTHALAMUS AND HYPOPHYSIAL STALK. Cajal (63), who gave the first clear description of the nerve fibers in the pituitary stalk, mentioned that some of these fibers penetrate between the epithelial cells of the pars intermedia. That nerve fibers enter this part of the adenohypophysis has been confirmed by many workers (31, 53, 56, 89, 162, 163, 171, 184, 335, 343, 344, 358, 397, 403, 412). Some authors indicate these nerve fibers to be relatively numerous (184, 397, 403), though most recent accounts describe them as being scanty (53, 171, 343, 344). Tello (397) described intercellular and intracellular endings for these fibers, but most workers have found only simple intercellular nerve terminals in the pars intermedia (184, 344).

The pars tuberalis appears to receive some nerve fibers from the hypothalamico-hypophysial tract as it lies in the median eminence and infundibular stem (65, 123, 171, 184, 344, 358). No very close relationship of nerve terminals to cells has been seen here, though a close neuro-vascular relationship has been noted (171). Vasquez-Lopez (412) described a close relationship between the nerve fibers and blood vessels in the narrow cortical zone of the median eminence and stalk, that is, the tissue adjacent to the pars tuberalis.

The innervation of the pars distalis is a much debated subject. An absolute (57, 163, 216) or relative (18, 53, 89, 171, 344, 397) lack of nerve fibers in this structure

has been reported many times, though a few workers describe the presence of numerous nerve fibers (184, 403). Particular attention is drawn to the careful work of Rasmussen (334), who gives a summary of the literature. Pericellular nerve nets have been described around the glandular cells of the pars distalis (53, 335), though as pointed out by Green (170), the published illustration of these nets (232) resemble the reticular nets previously described by Tello (397). Truscott (403) describes a well marked innervation to the pars distalis of the rat, passing forward from the neurohypophysis. This finding is not in accordance with previous observations on the same animal (53). Attempts have been made in the rat and rabbit to determine the origin of the nerve fibers in the pars distalis, by studying the glands of animals after section of the pituitary stalk and after cervical sympathectomy (53). Stalk section was found to eliminate most of the nerve fibers to all parts of the pituitary, while sympathectomy caused a partial reduction of nerve fibers in the pars distalis of the rabbit and no detectable reduction in the rat. Of much interest are the studies by Drager on the nerves to the pars distalis of the porpoise, bird (123, 124) and armadillo (125). In these forms a connective tissue septum intervenes between the infundibular process and pars distalis, thus limiting the route for transference of nerve fibers from the neurohypophysis to adenohypophysis. No hypothalamico-hypophysial nerve fibers were found in the pars distalis of the bird or porpoise, and only a few in the armadillo. These observations on the bird's pituitary are of especial significance, since gonadal activity in the bird appears to be particularly responsive to environmental change.

In summary, it may be said that the hypothalamic innervation of the adenohypophysis appears scanty if it constitutes a true secreto-motor nerve supply to the organ.

There is some experimental evidence that interference with the hypothalamus or pituitary stalk, by damage or stimulation, affects the activity of the adenohypophysis. This work will now be considered with respect to the actions this gland exerts through the gonadotrophic, lactogenic, thyrotrophic, adrenotrophic and diabetogenic hormones.

(i) *Hypothalamus and gonadotrophic hormones.* That damage of the hypothalamus may lead to genital atrophy is well known from clinical observations (quoted above) and from the early observations of Camus and Roussy (67), Bailey and Bremer (8) on dogs and Smith (383) on rats. Cushing (90) suggested Smith's results were due to interference with hypophysial blood supply or of interrupted nerve supply. More recent observations indicate that hypothalamic injuries may interfere with sexual functions through damage to the nervous structure underlying the integration of reaction patterns of sexual behavior, or through damage to some neural mechanism controlling the output of gonadotropic hormones from the pituitary. In the normal animal these two mechanisms are doubtless closely inter-related, but in experimental work they are frequently separated, studies of the first type often being performed on gonadectomized animals under controlled hormonal administration (12, 108).

Cahane and Cahane (60, 61) noted that hypothalamic lesions may produce genital atrophy in the rat. Following the observation that female cats with diabetes insipidus (produced by lesions in the hypothalamico-hypophysial tract) never bred in

the laboratory (141), Dey, Fisher, Berry and Ranson (116) and Dey (113, 115), found that lesions in guinea-pigs situated between the optic chiasma and pituitary stalk resulted in sexual disturbances. Some animals with lesions anterior to the mammillary bodies in variable sites showed normal oestrous cycles though many would not mate; others with lesions between the chiasma and median eminence showed a constantly open vagina, hypertrophied external genitalia and a lack of corpora lutea in the ovaries; while a third group with lesions at the junction of the hypothalamus and pituitary stalk showed genital atrophy with a constantly closed vagina. Loss of cyclical phenomena and genital atrophy were found more commonly after lesions in the median eminence than after lesions in other sites in the hypothalamus or in the pituitary stalk (114) or after hypophysial lesions (115, 118). In rats Hetherington and Ranson (217) describe obesity and sexual dystrophy following lesions in the tuber cinereum, as do Biggart and Alexander in dogs (21), and it has been stated (434) that an uninterrupted connection of hypothalamus, pars tuberalis and pars distalis is needed to ensure a normal production of gonadotrophic hormone. In spite of the claim of Diaz (119) that interference with the oestrous rhythm may be consequent to nonspecific trauma, such as nephrectomy, it would seem probable that lesions in the tuber cinereum which interrupt the oestrous cycle exert their effects by altering the hormonal output of the pituitary gland. The most direct route by which this effect could be mediated is the pituitary stalk, but data derived from experiments in which this structure has been sectioned are far from harmonious.

Section of the pituitary stalk in rats has been claimed to produce lengthened oestrous cycles (352), gonadal atrophy in male and female animals (46, 429, 430) and normal, prolonged or absent cycles (in the rats showing normal cycles, pseudopregnancy occurred following sterile coitus, 109, 110). Westman and Jacobsohn (429), who noted gonadal atrophy in the rat following stalk section, inserted a small silver plate between the cut ends of the stalk. In the rabbit stalk section has been reported to produce gonadal atrophy (196, 223, 432). Oestrous rabbits have been obtained following this operation (50, 54), however, though the normal ovulatory response to mating was absent in these animals. Brooks (50) investigated the blood vessels of the stalks in some of his operated animals and stated that they either had not been completely destroyed by the operation or had been reestablished in part. Westman and Jacobsohn (432) found that transection of the pituitary stalk in rabbits, within forty minutes of copulation, did not prevent subsequent ovulation, but injection of novocaine solution through the foramen opticum of unanesthetized female rabbits (433), which were then mated, did prevent the ovulatory response. Transection of the stalk in guinea-pigs may cause little interference with the reproductive system (106) or may be followed by normal, irregular or absent cycles (263). In dogs, silver clips applied to the pituitary stalk (280), or division of the stalk (121), caused sexual atrophy, while other workers (252) reported normal sex functions in the bitch following stalk section. Breckenridge (40) removed the hypophysial stalk and upper part of the pars anterior in nine dogs and found two bred normally, four showed anatomically normal reproductive tracts and three showed atrophic sex organs. There was no correlation between the amount of pars anterior tissue remaining and the state of the reproductive system. Brooks (51) makes brief mention of similar experiments on

monkeys. Dandy (99) describes a case of stalk section in a young adult woman that was followed by normal menstrual cycles, pregnancy, labor and lactation.

From this evidence it seems that lesions of the median eminence produce a greater disturbance of sex function than section of the pituitary stalk. It appears unlikely that all the cases of sexual dysfunction reported to follow lesions of the stalk can be explained in terms of concurrent damage to the median eminence or hypophysis. A more likely hypothesis is that lesions of the median eminence irreparably damage a neural mechanism which normally exerts a controlling influence over the activity of the adenohypophysis by vascular transmission down the pituitary stalk. These vessels might undergo regeneration following stalk section.

Stimulation has been performed by electrical and chemical means. The electrical method was first used by Marshall and Verney (291), who found strong diffuse stimulation of the brain of the oestrous rabbit was followed by ovulation and pseudopregnancy. Harris (195) showed pseudopregnancy followed similar stimuli applied to the rat. Attempts were made to localize the active focus, and it was shown that stimulation of the pituitary gland directly (196), the tuber cinereum (196 and see 51), the preoptic region (205) or posterior hypothalamus (196) produced ovulation in the rabbit. Stimulation of the tuber cinereum was still effective after cervical sympathectomy (196), though previous cauterization of the pituitary stalk prevents ovulation following brain stimulation (428). More recent attempts to delimit the focus (284) indicate that direct electrical stimulation of the pituitary gland is not followed by the follicular rupture which occurs after similar stimulation of the hypothalamus. The most reliable technique for investigating this problem is the remote-control method of stimulation, which renders unnecessary any anesthesia or concurrent trauma. With this procedure stimulation of the tuber cinereum is more effective in eliciting ovulation than stimulation of the infundibular stem or adenohypophysis (201). The fact that repeated electronarcosis of guinea-pigs (128) causes increased ovarian weight may be a further example of neural excitation of the adenohypophysis.

Intravenous administration of copper (134) and cadmium (131) salts, picrotoxin (292), metrazol (52) or various plant juices (38) is followed by ovulation in the rabbit. Other drugs and salts [pilocarpine, eserine, acetylcholine and adrenaline (291); strychnine, apomorphine, β-tetrahydro-naphthylamine, ergometrine, doryl, coriamyrtin (292); strychnine, insulin, benzedrine and ephedrine (52); salts of barium, cobalt, gold, iron, manganese, nickel, silver and zinc (131)] have been tried without success. It was first believed that the active substances might produce their effects by augmenting the action of the circulating pituitary hormones. Bischoff (22) then suggested that copper salts act on some nervous mechanism, and this idea is now supported by the evidence that transection of the hypophysial stalk, before or shortly after the intravenous injection of copper acetate, picrotoxin or metrazol inhibits the usual ovulatory response (52), and further, that the dose of copper acetate necessary to produce ovulation on injection into the region of the third ventricle is approximately $\frac{1}{200} - \frac{1}{300}$ of the intravenous dose (197). Intravenous administration of copper salts to oestrous rats is followed by pseudopregnancy (126). Pretreatment with oestrone makes it possible to induce pseudopregnancy or ovulation and pseudopregnancy, in met- or di-oestrous rats or anoestrous rabbits, respectively, by intravenous

injection of copper acetate (127). On the other hand, administration of progesterone to oestrous (post-partum) rabbits blocks the ovulation induced by copper salts (153). Injection of yohimbine is also reported to upset the normal sex rhythm in rats, producing a state of constant oestrus of three to nine days' duration (156).

Although intravenous injection of acetylcholine is not followed by ovulation in the rabbit (291), Taubenhaus and Soskin (394) claim that direct application of an acetylcholine-prostigmine mixture to the exposed pituitary glands of rats results in pseudopregnancy, and that similarly applied atropine prevents the pseudopregnancy which normally follows electrical stimulation of the uterine cervix. This evidence is taken by the authors to support the view that the adenohypophysis is normally excited by a humoral transmission from the hypothalamus via the hypophysial portal vessels in the pituitary stalk.

A further method whereby the adenohypophysis may be excited is by increased illumination. It is well known that increased illumination in midwinter causes female ferrets to come into full oestrus earlier than control animals (23). Ultraviolet light is more active in this respect than luminous rays (290). Ferrets in which the pituitary has been removed are non-responsive to extra illumination (220), and similarly, if the optic nerves are severed (24, 80), the cycles are freed from seasonal or photic influences or do not occur at all. It seems likely from this and other evidence that in the ferret, light stimulates the adenohypophysis by a neural, or neuro-humoral pathway from the retina. There is evidence that the nervous impulses pass from the optic tract either to the ventral nucleus of the lateral geniculate body or to the subthalamus by way of the accessory optic tracts (80). However, it is claimed (244) that the ferret lacks any accessory optic tracts. The final pathway serving this reflex activation of the pituitary remains obscure. It has been shown in the drake that light which has a marked effect in stimulating the testis of the normal bird is also effective after enucleation of the eyeballs. Gonadal stimulation was also obtained if the light was directed, by means of a quartz tube, on to the region of the pituitary gland (16, 17). These latter results are difficult to evaluate.

(ii) *Hypothalamus and lactogenic hormone.* It seems probable that nervous activity plays a part in the removal of milk from the actively secreting breast [the 'letting down' of milk from cattle (186)], but it is unlikely that it is directly concerned with the actual secretion of milk, which would appear to be under hormonal control by the pituitary gland. Stricker (390) showed that a mammary gland grafted into a rabbit's ear continues to secrete providing normal glands are being suckled. Also, lactation may occur normally in the completely sympathectomized cat (69), though some reports indicate that occasional impairment of lactation follows sympathectomy (7, 68, 380).

The idea was advanced by Selye and his co-workers (370, 373, 374, 375) that lactation is maintained in rats and mice by the act of suckling, which stimulates the adenohypophysis by some nervous pathway to liberate the lactogenic hormone. It was found that the stimulus of suckling prevents involution of the mammary glands, even those from which the nipple had been excised and no milk was removed. Weichert (424) found that the posterior breasts tend to be neglected in rats with small litters, but that regression of these is delayed by suckling of the more anterior glands.

Ingelbrecht (236) showed that severance of the spinal cord, in lactating rats, between the last dorsal and first lumbar segments resulted in failure of milk secretion and death of the young if only the posterior, anesthetic nipples were suckled, whereas if suckling of the anterior nipples was allowed milk secretion was maintained in all glands. The observations of Hooker and Williams (226) that mammary involution (after removal of the young) is retarded in all breasts by application of spirits of turpentine twice daily to all or some of the nipples also support Selye's theory. The influence of suckling on the lactogen content of the pituitary gland in rats and rabbits has been studied (300, 348).

The nervous pathway by which the stimulus of suckling excites the adenohypophysis is unknown. That lactation can occur normally in sympathectomized animals eliminates the cervical sympathetic system. The data concerning the hypophysial stalk are inconclusive. Desclin (111) and Herold (215) claim that transection of the stalk in rats causes failure of lactation in spite of functioning anterior pituitary tissue and continued suckling. Jacobsohn and Westman (243) found that the mammary involution which occurs in lactating rats following transection of the pituitary stalk is not so complete as that which occurs following hypophysectomy and state that their results do not uphold Selye's views. According to Dempsey and Uotila (110), however, stalk-transected rats may lactate normally. These latter results would indicate that the nerve fibers in the stalk are not essential to the mechanism.

(iii) *Hypothalamus, thyrotrophic and adenotrophic hormones.* Lichtwitz (267) suggested that the hypothalamus reacts to variations in thyroxine concentration, thus regulating and controlling the secretion of the thyrotrophic hormone from the pituitary. The evidence concerning this hypothesis is controversial.

Hypothalamic lesions have been stated to affect metabolic rate and thyroid histology. With lesions in the central hypothalamus, a fall in metabolism was observed in cats (29). Cahane and Cahane (62) noted that infundibular lesions may produce a histological picture of increased or decreased activity in the thyroid gland. From this and other evidence they postulated the presence of two centers in the infundibular region, one situated between the optic chiasma and stalk which excites the secretion of the thyrotropic hormone and the other in the group of tubero-mammillary nuclei which inhibits the secretion of this hormone. Grafe and Grunthal (166) noted a fall in metabolism of 35 per cent on destruction of the tuberal centers in a dog.

Section of the hypophysial stalk has been shown by most workers to have little effect on the metabolic rate or thyroid histology. Uotila working on stalk-sectioned rats found that the thyroid remained histologically normal (405), that it reacts with the usual atrophy to injections of thyroxine or with the usual compensatory hypertrophy after subtotal thyroidectomy (408), but that the normal hypertrophy evoked by a cold environment is lacking (405, 407). He suggests that the basic secretion of the thyrotrophic hormone is humorally controlled and independent of the pituitary stalk, but under certain environmental conditions this basic secretion can be modified by hypothalamic impulses which reach the hypophysis via the pituitary stalk. Brolin (46) records a decrease of thyroid activity following stalk section in rats and agrees with Uotila that cold exposure of such animals does not lead to the usual hypertrophy. Uotila (407) also found that the adrenal cortex was normal in weight

and histological appearance after stalk section, and that unlike the thyroid, some hypertrophy was elicited by the stimulus of cold. Brooks (50) remarks that the thyroid and adrenal glands of stalk-cut rabbits were generally of normal weight and showed no obvious histological abnormalities. Mahoney and Sheehan (280) described metabolic changes in puppies following stalk section as evinced by retardation of growth, sexual infantilism, adiposity, refinement of hair and sluggish general behavior. Since the pituitaries of these animals showed marked degenerative changes, they feel the condition may be explained by interference with the major blood supply to these glands. This work confirms earlier work on dogs, such as that of Dott (121), who divided the stalk and inserted a small platinum plate between the pituitary gland and tuber cinereum. He reported among other signs a lowered body temperature and degenerative changes in the thyroid gland. In stalk-sectioned monkeys, Mahoney and Sheehan (280) found a normal metabolic rate. Westman, *et al.* (435) divided the pituitary stalk in 14 rabbits and found that in 11 cases the thyroid gland exhibited the histological picture of diminished function. All their rabbits showed marked genital atrophy. These latter findings stand in contrast to those of Brooks (50).

The effect of stimulation of the hypothalamus or hypophysis upon thyroid or adrenal function has not been the subject of much study. Saxton and Greene (365) report that the stimulus of coitus causes the liberation of thyrotrophic and perhaps corticotrophic hormone from the hypophysis of female rabbits as well as the gonadotrophic hormone(s). Ellis and Wiersma (128) found that repeated electronarcosis of dogs and guinea-pigs caused hypertrophy of the thyroid and an increase in thyrotrophic hormone in the blood. Preliminary experiments by Green and Harris (172) have failed to demonstrate any increase in oxygen consumption in rabbits following electrical stimulation of various regions of the hypothalamus and hypophysis. Stimulation was applied by the remote control method for periods up to three hours daily for one week. It is possible that further work, involving longer periods of stimulation of various regions, may produce different results.

There is some evidence then, that the secretion of the thyrotrophic hormone is under hypothalamic control, mediated by the pituitary stalk.

(iv) *Hypothalamus and the diabetogenic hormone.* Hypophysectomy produces an increase in insulin sensitivity (229), a tendency to hypoglycemia and amelioration of a pre-existing pancreatic diabetes (228). Injection of anterior pituitary diabetogenic extracts may produce the reverse effects. It is of interest, therefore, with regard to hypothalamic control of the adenohypophysis, to consider how far these states may be duplicated by lesions or stimulation of the hypothalamus or hypophysial stalk. For a previous review of this subject see Long (270).

It has long been known that injury or puncture of the tuber cinereum may be followed by glycosuria (6, 67, 423). In many cases the glycosuria was of an acute nature, and it seems likely that it was induced by excitation of the sympathetic system rather than the pituitary gland.

An increased sensitivity to the action of insulin has been reported to follow hypothalamic lesions. Ingram and Barris (239) showed that lesions in the anterior, suprachiasmatic portion of the hypothalamus in cats produce an increase in insulin

sensitivity and a lowered sensitivity to adrenaline. Cleveland and Davis (82) found comparable changes in the reactions to insulin and adrenaline followed lesions in the medial hypothalamus, which included the paraventricular and ventromedial hypothalamic nuclei. Keller (250) similarly reports a markedly increased sensitivity to insulin in the majority of cats with large hypothalamic lesions, though there was little correlation between the extent and site of the lesion and the decrease in insulin tolerance. After hypothalamic lesions in many cats Brobeck (42) found insulin hypersensitivity in a very small proportion of cases. Barris and Ingram (13) found hypoglycemia followed lesions in the anterior hypothalamus, especially if the paraventricular nuclei were damaged. Similar hypoglycemic effects have been reported by Keller and his associates (97, 250, 253) and by Cleveland and Davis (82). Bloch (30) reports only a wider range of blood sugar than normal after destruction of the medial hypothalamic area in cats. Amelioration of a pancreatic diabetes by previous hypothalamic injury has been observed in cats by Davis, Cleveland and Ingram (100) and Cleveland and Davis (82). However, this finding was not confirmed by Brobeck and Long (43) in partially depancreatized rats. Ingram (237) in a recent report states that hypothalamic lesions greatly reduce the insulin requirments of pancreatectomized cats (9 out of 13), though there was no specificity as regards localization of the lesion. It would appear then that the cardinal signs of interference with carbohydrate metabolism which follow hypophysectomy may also be found with lesions in the hypothalamus. The possibility that the neural lesions exert their effects by pituitary mediation may explain the frequent association by different workers between their lesions and the paraventricular nuclei. Paraventriculohypophysial connections have now been proven to exist, and there is no evidence associating these fibers with the functions of the neurohypophysis.

It is generally agreed that section of the hypophysial stalk produces slight or no derangement of carbohydrate metabolism. Brooks (50) found that stalk-cut rabbits showed a normal blood sugar level and sugar tolerance, and there was no increased sensitivity to insulin. However, Westman and Jacobsohn (432) state that rabbits with transected stalks, in spite of normal blood sugar values, show the same extreme sensitivity to insulin after 24 hours' starvation as shown by hypophysectomized rabbits. Mahoney and Sheehan (280) noted no significant variation of blood sugar to follow stalk section in monkeys. These results have been confirmed and extended by Brobeck, Magoun and Ranson (44).

Stimulation of the lateral hypothalamus was shown by Himwich and Keller (222), and Magoun, Barris and Ranson (276) to produce hyperglycemia. As pointed out by Fulton (157) this acute phenomenon is probably brought about by activation of the sympatho-adrenal medullary system at the hypothalamic level. Lewy and Gassmann (266) obtained a more prolonged hyperglycemia lasting a few days following stimulation of the (perifornical) periventricular nucleus. In preliminary experiments Harris (201) has obtained a marked decrease in insulin sensitivity following prolonged stimulation (one to three hours daily for one week) of the tuberal region in rabbits. This glycotrophic effect is maximum after one week and slowly decreases after ceasing stimulation. These time relations may indicate a slow hypertrophy of the adrenal cortex as suggested by the work of Jensen and Grattan (168, 245) and

Heinbecker and Rolf (210). A possible explanation for the onset of diabetes mellitus during a course of metrazol therapy (173) may be hypothalamico-hypophysial excitation by this drug.

3. *Difficulties in Accepting a Direct Secreto-motor Innervation*

The outstanding features at the present time are the large bulk of evidence relating the activity of the adenohypophysis to neural control in general and hypothalamic control in particular; the conflicting evidence concerning interference with adenohypophysial function that follows pituitary stalk section; and the scarcity of nerve fibers in the pars distalis. There is no convincing evidence implicating the peripheral autonomic system with pituitary activity.

The pars distalis of the pituitary may, in general terms, be described as a gland under nervous control but lacking a nerve supply. This statement would appear to be most literally true for the bird where exteroceptive factors play a large part in regulating gonadal activity in spite of the apparent lack of nerve fibers in the pars distalis (124).

There is much experimental and clinical evidence that the hypothalamus is functionally linked with anterior pituitary activity. The most direct route by which this influence might be exerted is by means of the nerve fibers or blood vessels of the hypophysial stalk. It seems possible to eliminate the nerve fibers of the stalk from participation, since section of the hypophysial stalk may not interfere very markedly with the functions of the gland. However the portal vessels of the stalk, unlike the nerve fibers, may undergo repair following stalk section, and so reestablish humoral transmission of stimuli to the pars distalis.

4. *Evidence Concerning Neuro-vascular Transmission of Stimuli to the Adenohypophysis*

Popa and Fielding (338, 339) were the first to describe a true portal system of blood vessels in the pituitary stalk. From a study of human material they described a system of vessels collecting blood from all parts of the pituitary gland and uniting into larger trunks which could be traced up the stalk into the hypothalamus, where they were seen to break up into the 'secondary net' of capillaries. Wislocki and King (442) and Wislocki (437, 439) confirmed the presence of these vessels, but differed from Popa and Fielding in describing the upper limit of the portal vessels as situated in the expanded lower end of the tuber cinereum (known as the median eminence), the direction of blood flow as being downward and the blood draining entirely into the pars distalis. Since the median eminence is part of the neurohypophysis, the term 'hypophysial portal system' was substituted for 'hypothalamico-hypophysial portal system.' Green and Harris (171) reinvestigated the region in a variety of mammals (rat, guinea-pig, rabbit, dog and man) and largely confirmed the findings of Wislocki. They described small arterial twigs running from the internal carotid and posterior communicating arteries to supply a large vascular plexus situated between the pars tuberalis and median eminence. From this plexus very characteristic sinusoidal capillary loops penetrate the median eminence where they are surrounded by the wealth of nerve fibers of the hypothalamico-hypophysial tract. These capil-

laries then pass into large trunks which run down the hypophysial stalk, draining into the pars distalis where the blood is redistributed by the large sinusoids of the gland. The pattern differs in detail in different animals; in the human the upper system of capillary loops and tufts being present in the infundibular stem as well as the median eminence. Nerve fibers of the hypothalamico-hypophysial tract, besides being intimately related to the sinusoidal loops in the median eminence, pass in moderate numbers into the pars tuberalis, where they may be seen associated with the vascular plexus.

Harris (196), Hinsey (223), Brooks (50), Taubenhaus and Soskin (394) and others, have mentioned the possibility that neural control of the adenohypophysis is humorally transmitted from the neurohypophysis to the pars distalis. From the anatomical studies mentioned above, it seems possible that nervous stimuli might cause the liberation of some substance into the capillary sinusoids of the median eminence, this substance then being transported via the hypophysial portal vessels to excite or inhibit the pars distalis. Evidence concerning this view has been summarized by Harris (198) and Green and Harris (171).

(i) Ovulation in the rabbit normally occurs 10 hours after coitus. If hypophysectomy is performed within one hour of coitus, ovulation does not occur (132). It appears that the pituitary must remain intact for one hour following coitus for the secretion of sufficient gonadotropic hormone to produce follicular rupture. Since the nervous stimulus of mating takes only a few minutes, this may indicate that one link in the chain of events is a slow, humoral excitation of the adenohypophysis. Westman and Jacobsohn (432) claim that transection of the stalk 40 minutes after coitus does not prevent the subsequent ovulation, so it would seem that the delay occurs in the pituitary gland itself.

(ii) Markee, Sawyer and Hollinshead (284) have shown that ovulation is more easily elicited by electrical stimulation of the hypothalamus than of the hypophysis. At the time of their report the same result had been obtained from experiments in which the hypothalamus or pituitary gland had been stimulated in rabbits by the remote control method (Harris, 201). Direct stimulation of the adenohypophysis or the hypothalamico-hypophysial tract in the infundibular stem for 15 hours, in hourly periods distributed over 5 days, failed to elicit ovulation, whereas similar stimulation of the tuber cinereum for three minutes (a time period approaching that of the normal stimulus of coitus) was followed by an ovulatory response. If the adenohypophysis is devoid of a direct secreto-motor nerve supply, it is possibly not excitable by direct electrical stimulation (cf. electrical stimulation of the denervated adrenal medulla, 70).

(iii) The different effects recorded after section of the hypophysial stalk (40, 50, 54, 106, 109, 110, 111, 121, 196, 215, 223, 252, 263, 280, 352, 405, 434, 435) may be explained by variations in degree of regeneration of the hypophysial portal vessels. The observation of Brooks (50) that the hypophysial portal vessels of his stalk-cut rabbits either had not been completely destroyed by the operation or had become reestablished in part is of interest in this connection. Those workers who have taken the precaution of placing small metallic plates between the divided ends of the stalk (121, 429) have described gonadal atrophy following the operation. India ink

injections of the vascular tree of rats with divided stalks (201) indicate that regeneration of the hypophysial portal vessels may occur. The observation of Westman, Jacobsohn and Hillarp (434) that it is the connection of the pars tuberalis with the hypothalamus and not that of the infundibular process which is of significance in preserving a normal cytology of the pars distalis and a normal output of gonadotrophic hormone indicates the importance of the structure bearing the portal vessels.

(iv) The results of Dempsey (106), Dey (114) and Leininger and Ranson (263) indicate that a greater disturbance of the oestrous cycle in the guinea-pig may follow a lesion in the median eminence rather than section of the hypophysial stalk. This might be due to the former producing a complete, irreparable denervation of the median eminence and sinusoidal capillaries, while stalk section allows the possibility of vascular repair.

(v) In some animals, such as the whale (Wislocki and Geiling, 441), porpoise (Geiling, Vos and Oldham 160), sea-cow (Oldham, McCleery and Geiling, 322), armadillo (Oldham, 321) and Indian elephant(Wislocki, 440) the infundibular lobe is separated from the pars intermedia, or pars distalis if the intermedia is lacking, by a connective tissue septum derived from the dural capsule of the gland. This septum eliminates the main pathway for nerve fibers between the infundibular process and pars distalis. However, from the literature it seems these animals have a pars tuberalis in contact with the median eminence and probably a portal system of vessels. The pars distalis of the porpoise is said to be free of any nerve fibers (123), but this animal shows a very well marked hypophysial portal system originating in the median eminence, passing ventrally in front of the connective tissue septum separating the two lobes and ending in the pars distalis (200).

(vi) Taubenhaus and Soskin (394) state the pars distalis of the rat may be stimulated by local application of a prostigmine-acetylcholine mixture. They adduce this fact, among others, as evidence for the theory of humoral control of the adenohypophysis.

At the moment there is insufficient evidence available to estimate the functional importance of the few nerve fibers which enter the adenohypophysis or of the hypophysial portal vessels. Further information regarding this basic problem of endocrine physiology is eagerly awaited.

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FERTILIZATION AND IMMUNITY[1]

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IN THE PRESENT ARTICLE an attempt is made to review the work on fertilization which, for the most part, correlates the reactions involved with those of immunological processes. Such correlation has been indicated by many earlier writers. Thus Loeb (124) pointed to the similarity of fertilization to phagocytosis, although he did not go much beyond the superficial resemblance of these two processes. A closer correlation is derived from the presence in eggs and sperm of specific substances that interact in the manner of antigens and antibodies. F. R. Lillie (107–118), who provided the first substantial demonstration of one of these substances, which he termed fertilizin, also attempted a description of the fertilization process in immunological terms. The correlation becomes even stronger as a result of recent work showing that an enzymatic lytic agent termed hyaluronidase may be involved in both fertilization and infection.

In many respects present-day knowledge of immunological reactions is further advanced than is that of the reactions of fertilization. So, to the extent to which the two may be correlated, immunological principles may help explain various features of fertilization. However, it can be anticipated that the relation will not remain one-sided and there are, in fact, already indications of the directions from which studies on fertilization may contribute to our knowledge of immunity.

There are four specific interacting substances, or groups of substances, of eggs and sperm which will be considered in this review. These have been designated fertilizins of eggs, antifertilizins of sperm, antifertilizins of eggs and lytic agents of sperm. Hartmann (64) has introduced the term gamone to designate the substance derived from eggs (gynogamones) and sperm (androgamones). However, the hormonal analogies of that terminology seem at least as objectionable as the implication of Lillie's terms. The term ovulin, suggested by Dalcq (26), and a corresponding spermulin might be preferable, but since the fertilizin terminology is so widely known it would be best to adhere to it for the present. When the various substances become better characterized, designation can, of course, be more readily made on the basis of specific chemical action and properties. Thus one of the lytic agents obtained from sperm is now termed hyaluronidase because of its enzymatic action on a polysaccharide known as hyaluronic acid (134).

FERTILIZIN

A. EFFECTS. Several early embryologists in studies on fertilization in echinoderms had noted effects of egg extracts on spermatozoa of the same or foreign species. In

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particular, agglutinative and other effects had been observed by von Dungern (231), Buller (6), Schücking (192) and DeMeyer (29) but little attention was paid to these until F. R. Lillie (107–116) initiated an extensive series of investigations of the subject. Lillie showed that the supernatant sea water (termed egg water) from a suspension of ripe, uninjured, sea urchin eggs would, when added to a suspension of sperm of the same species, cause an agglutination of the spermatozoa. Another effect of the egg water that he noted consisted in an increase in the activity of the spermatozoa. A third effect, namely chemotaxis of spermatozoa by egg water, has also been claimed by various workers (see 144, for critical review) but this still requires adequate demonstration. A fourth effect of egg water consists in inducing spawning of ripe males of certain species of animals. While the term fertilizin has been used to designate the substance or substances in egg water having all of these as well as other related effects, the agglutinative action is the one most generally employed in the tests.

1. *Agglutination.* The agglutinating action of egg water upon spermatozoa of the same species has been observed in a large number of species of animals, particularly among the echinoderms A fairly complete list of species in which such agglutination has been reported is given in table 1. Certain of these require some qualification. Thus, the report of agglutination in the fish Fundulus and the frog by Glaser (52) is contained simply in a parenthetic sentence, so Montalenti and Schartau's (141, 191) account in the Lamprey is the first real description of the phenomenon in vertebrates. In Chiton tuberculatus, Southwick (195) reports negative results with egg water but confirms Crozier's (25) finding of agglutination of spermatozoa that have passed through the mantle cavity of a male or immature female. In the starfish several workers (190, 91, 204, 133) find no agglutinative effects of ordinary egg water. Negative results are, of course, not frequently reported in the literature since failure can often be attributed to lack of sufficient trial under proper conditions. However, in addition to the cases mentioned above, it seems reasonably certain that agglutination of sperm does not occur with ordinary egg water in several species of chitons, the abalone and Cumingia (190) nor in Urechis campo (204). Even among the echinoids the phenomenon is evidently not universal since Vasseur and Hagström (230) report failure of isoagglutination in Brissopsis lyrifera.

Lillie (114) was aware of the fact that in many species of animals the agglutinative action of egg water was lacking. Nevertheless, he assumed that an analogous fertilizin was present in such species but in insufficient concentrations or in altered conditions, so that the readily visible agglutination was not apparent. Support for this view will be presented below in a discussion of 'univalent' fertilizin.

In the agglutination reaction in the sea urchins the sperm ordinarily unite mainly by their heads (114, 36). However, tail to tail and head to tail as well as head to head unions have been reported in other groups of animals such as the chiton Katharina tunicata (190) and the keyhole limpet Megathura crenulata (202). They may occur in sea urchins, too if the sperm suspensions are allowed to age (190). The types of reaction obtained with egg water are quite similar in appearance to those observed in the agglutination of sperm in antisera, such as figured by Henle, and Chambers (78) for bull spermatozoa. The latter authors have demonstrated separate antibodies for

head and tail antigens in such antisera produced in rabbits. For the action of the egg waters the evidence (202) indicates that only one kind of agglutinin is involved. However, the essential absorption tests with separate head and tail fragments of spermatozoa have not as yet been performed.

TABLE 1. SPECIES OF ANIMALS IN WHICH AGGLUTINATION OF SPERM BY EGG WATER HAS BEEN DESCRIBED

Echinoids
- Arbacia punctulata (Lillie, 1912, 1913)
- Arbacia pustulosa (Elster, 1935; Hartmann *et al.*, 1940)
- Strongylocentrotus purpuratus (Loeb, 1914; Lillie, 1921; Tyler, 1940)
- Strongylocentrotus franciscanus (Loeb, 1914; Lillie, 1921)
- Paracentrotus lividus (Just, 1929; Elster, 1935)
- Echinus microtuberculatus (Just, 1929; Elster, 1935)
- Echinus esculentus (Carter, 1932)
- Psammechinus miliaris (Vasseur and Hagström, 1946; Runnström and Lindvall, 1946)
- Echinometra subangularis (Southwick, 1939)
- Sphaerechinus granularis (Elster, 1935)
- Echinocardium cordatum (Elster, 1935; Vasseur and Hagström, 1946; Runnström and Lindvall, 1946)
- Echinarachnius parma (Just, 1919)

Asteroids
- Asterias forbesii (Glaser, 1914)
- Asterina pectinifera (Nomura, 1924)

Amphineuran mollusks
- Chiton tuberculatus (Crozier, 1922)
- Katharina tunicata (Sampson, 1922)

Gastropod mollusks
- Megathura crenulata (Tyler, 1940)

Pelecypod mollusks
- Ostrea virginica (Glaser, 1921)
- Ostrea circumpicta (Terao, 1926)
- Solen ensis (von Medem, 1942)
- Pecten varius (von Medem, 1942)

Polychaet annelids
- Nereis limbata (Lillie, 1912, 1913)
- Platynereis megalops (Just, 1915, 1930)

Cyclostome
- Lampetra fluviatilis (Montalenti and Schartau, 1941)

Fish
- Fundulus heteroclitus (Glaser, 1921)
- Salmo irideus (Hartmann, 1944, 1947)

Amphibia
- Rana pipiens (Glaser, 1921)

An interesting feature of the agglutination reaction, as it occurs in most species of sea urchins, is its spontaneous reversibility. Lillie (114) and Just (91) considered this feature to be characteristic of isoagglutination reactions as contrasted with the more permanent union they observed in the heteroagglutination of sperm by egg water or body fluids of a foreign species. However, Lillie (109, p. 552) noted that in Nereis "the agglutinations are essentially permanent" and irreversible isoagglutination has

also been described in Katharina (190), in Megathura (202) and even in a sea urchin, Echinocardium cordatum (230, 183). Spontaneous reversal cannot, then, be regarded as a criterion for typical isoagglutination. It is a phenomenon that is not ordinarily encountered in serological reactions and, therefore, requires further consideration. This will be given below in the section of 'univalent' fertilizin. Apart from the occurrence of spontaneous reversal in sea urchins, the agglutination of spermatozoa by egg water is essentially a serological type of reaction as Lillie (114) indicated. Even such features as the zone phenomena are manifested (202, 196) and other evidence, to be presented below, points to basic similarity to antigen-antibody reactions.

2. *Activation.* The stimulating action of egg water on the motility of spermatozoa has been noted in most of the species listed above, including many of those not exhibiting agglutination. Most of the observations on increased activity of sperm are highly subjective, so that it would be difficult to make a very useful list. When the spermatozoa are normally highly active, as is the case with freshly shed dilute sperm from fully ripe males of most species of sea urchins, an activating effect of egg water is not readily discernable, as Lillie (114) noted. However, when they are in a relatively quiescent condition, as a result of aging or special treatment, tests of activation by egg water may be made and several workers have reported such action in sea urchins as well as in other animals. Accompanying such increased activity increase in rate of oxygen uptake of the spermatozoa under the influence of egg water has also been noted in certain species. Thus Gray (59) presents data on the initial respiratory rate of Echinus esculentus sperm in egg water that ranged in different tests from 210 to 425 per cent of the control values. In another species, Echinus miliaris, no such increases are noted (59, 60), but rather a retardation in the normal decline in respiratory rate that occurs with time. The difference in response correlates with difference in activity of the sperm suspensions in the two species, those of E. miliaris being ordinarily much more active than those of E. esculentus. In Arbacia, Hayashi (72) notes a decrease in the rate of oxygen uptake under the influence of egg water. He attributes this to the agglutinating action of the egg water. However, it has been shown (e.g., Haurowitz and Schwerin, 70) that cells may be agglutinated by antibodies without reduction of their respiratory rate. In another species of sea urchin, Strongylocentrotus purpuratus, tests in this laboratory (196) have shown no increase in respiratory rate upon the addition of egg water to the suspensions. On the other hand, in the keyhole limpet, Megathura crenulata, the rate increases to between 300 and 400 per cent (221).

3. *Chemotaxis.* Various early investigators have presented evidence both for and against the existence of an attractive influence of eggs or egg water for spermatozoa. In an extensive review of the subject Morgan (144) concluded that the evidence presented in favor of such chemotaxis does not actually support it. The principal difficulty consists in distinguishing between chemotaxis and the trap action type of behavior described by Jennings (84). Although there has been a number of more recent reports concerning chemotaxis (21, 66–69, 230, 232), in none of these does this difficulty appear to be resolved.

4. *Spawning.* In marine animals the shedding of gametes by animals of one sex in

response to the presence of gametes of the opposite sex is quite commonly observed. Such stimulation of spawning has been reported in chitons (73), in nereids (117, 85), in sea urchins (43), in oysters (46–49, 158) and in mussels (250). In the case of the nereids and the oysters it has been shown that egg water alone will induce spawning in males of the same species. Properties of the spawning-inducing agent and its relation to the agglutinin present in egg water will be considered below.

B. CHEMICAL PROPERTIES. 1. *Agglutinin.* The source of the agglutinative agent of the egg water has been the subject of some controversy. Lillie (110, 116) showed that the gelatinous coat of the sea urchin egg contains the agglutinin in high titer but considered it to be continuously produced by the ripe unfertilized eggs as they remained in sea water. Loeb (122) on the other hand considered the agglutinin to be simply particles of the gelatinous coat. While Lillie's experimental data have been confirmed, recent evidence of Tyler and Fox (222, 223), Tyler (202, 204), Evans *et al.* (38), Hartmann *et al.* (69), Vasseur and Hagström (230) and Runnström and Lindvall (183) identifies the agglutinin with material of the gelatinous coat that goes into solution as the eggs stand in sea water, and rules out any continuous production or secretion by the eggs after they are shed from the ovary.

Lillie (110) showed that the sperm agglutinin of Arbacia egg water, by its failure to dialyze through a collodion membrane, is of large molecular size. Glaser (50) and Woodward (247) failed to obtain, with Arbacia egg water, the common protein tests except for a weak xanthoproteic test. More recently Tyler and Fox (222, 223) obtained positive xanthoproteic, biuret and Millon's reactions with agglutinin preparations of Strongylocentrotus and of Megathura prepared by dialysis and ammonium sulfate precipitation of 'uncontaminated' egg water, while Kuhn and Wallenfels (95) obtained positive ninhydrin reaction with an agglutinin (termed by them Hilfsträger) preparation of Arbacia. From various other chemical properties, including especially inactivation by crystalline preparations of the proteinases trypsin and chymotrypsin, it has been concluded by Tyler and Fox (222, 223) that the agglutinins of the sea urchin and the keyhole limpet are of the nature of proteins. However, the nitrogen content is considerably lower than the values usually obtained with simple proteins. Values around five per cent have been obtained in Megathura and Strongylocentrotus (223) and 8.0 to 8.5 per cent in Arbacia (95). With agglutinin preparations of Strongylocentrotus that show a single electrophoretic boundary and from which the organic material can be practically completely absorbed by the sperm, nitrogen values of 5.6 to 5.7 per cent have been obtained (221).

It has been suggested by Carter (11) that the agglutinin is a substance related to thyroxine which, he finds, causes an irreversible agglutination of sperm of sea urchins and starfish. Possibly some such substance may be liberated from egg water but this remains to be adequately demonstrated.

For the preparation of the agglutinin of Strongylocentrotus purpuratus the following procedure has been found (221) to be most effective. The washed egg suspension is acidified to pH 3.5 which dissolves the gelatinous coat of the egg. A 20 per cent suspension of eggs thus yields an egg water having a titer (maximum dilution giving agglutination when tested with an equal volume of one per cent sperm suspension) of about 1000. Addition of 4 cc. 1 N NaOH per 100 cc. of the egg water completely

precipitates the agglutinin along with certain sea water salts. The precipitate is taken up in 3.3 per cent NaCl. The agglutinin is again practically quantitatively precipitated by the addition of five volumes of 95 per cent alcohol to four volumes of the saline solution in the cold. Larger amounts of alcohol give no increase in the amount of precipitate or agglutinin. The agglutinin can be reprecipitated from saline solution with alcohol or with ammonium sulphate, which Woodward (247), Tyler and Fox (222, 223) and Kuhn and Wallenfels (95) showed to be an effective salting-out agent. However, electrophoretically homogeneous preparations are obtained without the use of ammonium sulphate. An egg water with an agglutinative titer of about 4000 yields approximately one mgm. of alcohol-precipitable material per cc.

Runnström, Tiselius and Vasseur (189) have shown that the material of the gelatinous coat of Psammechinus eggs is of pronounced acidic character migrating electrophoretically to the anode at pH 6.0 to pH 3.8. Ultracentrifugation of a solution containing 0.26 mgm. N per cc. showed a main component with a sedimentation constant of 2.9×10^{-13} varying with concentration in the manner characteristic of elongated, gel-forming molecules. In the absence of measurements of double refraction of flow, estimates of the molecular weight cannot readily be made. They also report a positive carbohydrate reaction of the material by a carbazole method. Their solutions did not exhibit agglutinating action, which is weak and often lacking in Psammechinus (cf. 230). This may, however, be due to condition of the sperm or some modification of the material, so it is not unreasonable to assume that their data may apply qualitatively to agglutinins of other species.

With hydrolyzed (three hours boiling in one N HCl) agglutinin preparations of Strongylocentrotus carbohydrate reactions have been obtained (221) with Molisch's and related reagents. With phenylhydrazine an osazone having the crystal form of galactosazone has been obtained. The hydrolyzed agglutinin yields reducing sugars amounting to about 15 per cent (calculated as glucose) of the original material present. Using Kunitz's (99) method, glucosamine has also been found in the hydrolysate by Krauss (93) in amounts equivalent to about four per cent of the original material. In Arbacia, Kuhn and Wallenfels (95) find about two thirds of the nitrogen in the supernatant of a vigorously hydrolyzed agglutinin preparation to be amino nitrogen. It is evident that the agglutinin of sea urchins contains both sugars and amino acids and may, then, be considered a glycoprotein.

The agglutinins have been found to be heat labile (114). The inactivation time is a function of pH and differs considerably in different species (223). Both Strongylocentrotus purpuratus and Megathura crenulata agglutinins are most stable around pH 4 in sea water but, whereas the former is half inactivated in 15 minutes at 100°C., the latter requires over 24 hours. A similar difference is found in their rate of inactivation by trypsin and chymotrypsin. No coagulation occurs when the fertilizins are inactivated by boiling and the initial loss of agglutinating activity does not involve destruction of ability to combine with sperm.

2. *Activating agent.* In 1939 Hartmann, Kuhn, Schartau and Wallenfels reported that the sperm-stimulating agent of Arbacia pustulosa egg water is identical with a pigment known as echinochrome, which Ball (4) and Lederer and Glaser (102, 56) had

been able to isolate in crystalline form from sea urchins. This pigment occurs in the test and elaeocytes of sea urchins, but only in the genus Arbacia is it also found in the eggs. It is not surprising, then, that no activating influence of echinochrome, nor of the related spinochrome, was found (201) in experiments with Strongylocentrotus purpuratus. However, in Arbacia punctulata negative results have also been reported (21) and it has been suggested that the original stimulating action may have been caused by alkalinity of the solutions. Kuhn and Wallenfels (94–97, 233–238) have determined the structure of echinochrome and related pigments and find them to be substituted naphthoquinones, the first examples of this class of pigments to be found in animals. Although it is closely related and may be readily converted into compounds having vitamin K activity, echinochrome A (3,5,6,7,8-hydroxy, 2-ethyl, 1:4-naphthoquinone) itself was found (98) to have no antihemorrhagic action in chickens. Possibly it may act in such manner in sea urchins. According to Shapiro (193) it is partially released into sea water from fertilized but not unfertilized eggs.

Kuhn and Wallenfels (95) showed that, when eggs of Arbacia pustulosa are deproved of their gelatinous coat, frozen, thawed and extracted with sea water, the echinochrome is obtained in combination with a nondialyzable carrier. According to Hartmann *et al.* (69) this 'binary complex' has no stimulating or agglutinating action on spermatozoa but active echinochrome can be split off by means of dilute acid. If the eggs are not deprived of their gelatinous coat the extract contains the latter material bound to the binary complex as a cocarrier (Hilfsträger) to form a tertiary complex which, they find, has both activating and agglutinating action. Upon dialysis against distilled water the binary complex precipitates out leaving the cocarrier in solution. The latter shows agglutinating but no stimulating action on the sperm. Cornman (21) has confirmed the stimulating action of a similarly prepared tertiary complex in Arbacia punctulata but attributes its activity to the protein portion. However, he and also Vasseur and Hagström (230) report that the activating agent is at least partially dialyzable. On the other hand Kuhn and Wallenfels (95) report that the activating agent in egg water fails to pass through cellophane even after several days dialysis against sea water. In Strongylocentrotus and Megathura, Tyler and Fox (222, 223) also find the activating agent to remain associated with the agglutinin upon precipitation with ammonium sulphate and dialysis. Clowes and Bachman (19) had noted that a sperm-stimulating agent can be distilled from egg water by boiling. Cornman (21) confirms this and adds that the distillate becomes inactive very quickly at pH 6. Possibly some nonspecific agent such as ammonia may be involved. Thyroxine has been shown by Carter (9–12) to activate unripe sperm and he suggests that a related substance in egg water is the effective agent.

On the whole the evidence indicates that the activating agent is normally bound to the agglutinin and may perhaps be split off as a smaller molecular constituent under certain conditions, but its chemical identity does not appear to be unequivocally established.

3. *Spawning-inducing agent.* There were indications in the early work of Lillie and Just (117) that the agent in Nereis egg water that induces spawning of the males might be the same as the sperm-agglutinin which it resembled in being nondialyzable,

similarly inactivated by heat, species specific and bound by sperm. Townsend (199) has suggested that the agent may be glutathione but her evidence does not appear very convincing.

Galtsoff (46–49) has presented some data concerning the nature of the spawning-inducing agent in the oyster egg water without making any direct comparison with the sperm agglutinin. He finds it to be relatively heat stable and dialyzable. Eggs of the pelecypod mollusks and of the starfish, but not the sea urchin, will induce spawning of male oysters which will respond occasionally also to oyster sperm. Female oysters will respond to the presence of species-sperm or a thermolabile constituent that is insoluble in sea water and soluble in alcohol and benzene, but not to sperm of other pelecypods. The male oysters also respond to a variety of chemical substances including thyroxin and glutathione, but Galtsoff (49) does not conclude that either of these are the active agents in egg water.

Nelson and Allison (158) have shown that the receptors, for the stimulation of spawning of the male oyster by eggs, are located on the surface of the demibranchs and that the shedding of sperm results from a relaxation of the sphincter of the distended sperm duct. A new hormone-like substance, termed diantlin, was discovered earlier by Nelson (1935) in oyster sperm. When added to the incurrent water this agent causes a great increase in the rate of water pumpage through the oyster's gills. Nelson and Allison show this effect to involve relaxation of the adductor muscle, enlargement of the gill openings and increased ciliary activity. This effect is not given by sperm of other mollusks or annelids, nor by various substances that induce spawning of the male oyster. By means of various tests, including inactivation by trypsin, they show diantlin to be of a protein nature. Upon acidification (0.1 N HCl) they obtain separate basic protein and nucleoprotein (nucleic acid ?) fractions which, by themselves, are inactive but which can be recombined to give the active agent again. Diantlin is evidently quite different from the alcohol soluble substance of sperm which, according to Galtsoff, induces spawning of the females. It could, however, be related to the antifertilizin of sperm which is discussed below (p. 193).

C. 'UNIVALENT' FERTILIZIN. 1. *Spontaneous reversal of agglutination.* In the usual serological reactions with immune sera or specific normal sera, agglutination ordinarily persists as long as the cells remain intact. While this is the case, too, for the action of egg water on sperm in certain species of animals, spontaneous reversal occurs in various species of sea urchins. If interpretations of the mechanism of agglutination reactions are to apply to these reactions, too, the reversal must be taken into account. There is now fairly general acceptance of the mutual multivalence theory of Heidelberger (74, 75) and Marrack (128) which recent evidence of Pauling *et al.*, (see 167, 169) strongly supports. Briefly, the theory postulates that antigen and antibody are both multivalent with respect to their structurally complementary combining groups. Agglutination or precipitation, then, results from the building up of large aggregates by combination of each molecule of antigen or antibody with more than one molecule of the other. 'Univalent' antigen (e.g., a single hapten) or 'univalent' antibody, although still capable of reacting specifically, would be incapable of forming such aggregates and would inhibit agglutination or precipitation of the 'multivalent' reagents.

After spontaneous reversal in the sea urchin following agglutination by excess egg water the spermatozoa cannot be reagglutinated (114), although they remain intact and active. It has also been shown (223) that the agglutinin is considerably more stable in species like Megathura in which the agglutinates persist. Several interpretations of the reversal, consistent with the mutual multivalence theory, have been suggested by Tyler (204), including the view that the fertilizin molecules are split by action of the sperm leaving essentially univalent fragments combined with the antifertilizin of the sperm. This view is supported by experiments in which material having the properties expected of 'univalents' is obtained.

2. *Production of 'univalent' fertilizin.* When sea urchin egg water is exposed to the action of heat, proteolytic enzymes, ultraviolet or X-radiation to the point where it no longer agglutinates sperm, it still is found to contain material capable of combining specifically with the sperm (204, 206, 132). Such solutions inhibit agglutination of the sperm by untreated egg water. The altered agglutinin is still nondialyzable and is absorbed by species sperm. It also impairs the fertilizing power of sperm as does untreated agglutinin (109, 204). By analogy with inhibiting antibodies the altered fertilizin is termed 'univalent'. Sea urchin fertilizin does not require very drastic treatment (compared, for example, with the hydrolysis of proteins and polysaccharides) to effect conversion into the 'univalent' form. Thus 20 minutes boiling at pH 8 is effective. Evidently it is not necessary to rupture strong bonds for this purpose. In the spontaneous reversal of agglutination it is, presumably, these same bonds that are broken. It is not inconceivable that the sperm accomplish this mechanically, but it seems more reasonable to suppose that they do it enzymatically. The fact that relatively inactive or aged sperm do not exhibit spontaneous reversal can be interpreted to support either interpretation, depending on additional evidence. The possible presence of a depolymerase in sea urchin sperm will be discussed below.

3. *Species lacking sperm-agglutinin.* Egg waters of the starfish, Patiria miniata, and the gephyrean worm, Urechis caupo, which do not ordinarily cause agglutination of homologous sperm, were found by Tyler (204) to reduce the fertilizing power of the latter. That such egg waters actually contain material that may be termed fertilizin has been shown by the work of Metz (133). He found that the addition of some nonspecific adjuvant (from hen's egg white or the serum of lobster, fish, rabbit or chicken) resulted in agglutination of starfish sperm with homologous egg water. In tests with egg waters of three species of starfish no cross agglutination was obtained. The adjuvant of hen's egg white was found to be a heat stable, nondialyzable substance, insoluble in acetone or alcohol. Without egg water it activates but does not agglutinate the sperm. From various tests, including the obtaining of an agglutination-inhibiting substance by ultraviolet irradiation of the egg water, Metz concludes that it is the sperm which is effectively 'univalent' and that the adjuvant exposes more combining groups on it. Apart from the question as to whether it is the sperm or egg water that is 'univalent', this work supplies strong support for Lillie's (114) view that fertilizin is present in species in which sperm-agglutination by egg water is not ordinarily obtained. In addition it provides an important technical means of investigating the specific interacting substances of such species.

4. *'Univalent' antibodies.* The work on 'univalent' fertilizin has its counterpart in the field of immunology. Analogous conversion of agglutinins or precipitins of immune sera into 'univalents' has been obtained by various workers (see 212 for references) by means of such agents as heat, diazo compounds, formaldehyde and photooxidation. In these cases the effect is also interpreted (209–213) as due to a splitting of the antibody molecules and, in addition, a reassociation of the 'univalent' fragments with similarly split nonantibody protein of the serum. The 'univalent' antibodies retain their original specificity and, in the case of an antitoxin (229), may still function as a protective agent. Such treated antisera are found to have, in general, reduced antigenicity and may thus be of some practical value in connection with serum sickness, etc. Analogous to the occurrence of nonagglutinating fertilizin in some species of animals, immune antibodies have also been found to be formed as 'univalents' in certain cases (20, 163, 77). Of recent interest are the blocking ('univalent') antibodies found in Rh antisera (242, 177, 40, 106). In the presence of serum albumin these antibodies are found (243, 30, 27) to be capable of agglutinating Rh positive cells. This again resembles the situation in the starfish where an adjuvant is needed to enable the egg water to agglutinate homologous sperm (133).

D. Specificity. The sperm-agglutinin of egg water is fairly specific for homologous sperm. Cross-reactions may occur between related species and, while these are generally weaker than the homologous reactions, occasionally they may be even stronger (see 36, 230). In addition there are instances of cross-reactivity between remotely related species, such as the agglutination of Nereis sperm by Arbacia egg water (109). The Nereis sperm are also agglutinated by Arbacia body fluid which has no agglutinating action on homologous sperm. Lillie (110) showed that the heteroagglutinating activity of Arbacia egg water could be removed by absorption with Nereis sperm, or even sperm of the teleost fish Ctenolabrus, leaving the isoagglutinin intact. Just (87) has also presented evidence for a separate heteroagglutinin to account for the action of Arbacia egg water on Echinarachinus sperm. However, Elster (36) finds in cross-tests among echinoids, no indication of distinct heteroagglutinins.

Heteroagglutinins are of quite widespread occurrence in the normal serum and body body fluids of vertebrates (see 241, 101) and invertebrates (82, 225, 215). It is not surprising then that cross-reactions are found (e.g., 109, 110, 87, 91, 190, 57, 69, 184) to occur between spermatozoa and foreign egg waters, body fluids and spermatozoa or their extracts. A recent investigation by Tyler (215) of the body fluid and seminal fluid of 12 species of invertebrates showed these fluids to have agglutinating action on the spermatozoa or blood cells of some or most of 34 species of animals. In lobster serum, which normally acts on the sperm or blood cells of a wide variety of animals throughout the animal kingdom, at least ten distinct, relatively class-specific, agglutinins have been demonstrated, by Tyler and Metz (224, 225) by means of absorption tests. Cells (blood cells or spermatozoa) of one species remove the agglutinins for other species of the same group, but not those for species belonging to other groups. The ten different heteroagglutinins of lobster serum are evidently (227) represented by a single protein component that is electrophoretically homogenous and of large molecular size. In starfish body fluid at least four distinct heteroagglutinins are

found (215). In work on various immune or normal sera it has been established (see 1, for references) that antibodies with different specificities may be represented by a single, electrophoretically homogenous, protein component. The serological differences evidently do not involve changes in such physicochemical properties. The differences have come to be regarded (see Pauling, 167, 169) as differences in spatial configuration rather than of composition, the specificity of interaction being dependent upon the degree of structural complementariness of antibody and antigen. In the case of heteroagglutinins of such wide reactivity as described here, it has been suggested (225, 215) that the reactants are not complementary in detail but in the overall structure of large regions. Substances of large molecular size would presumably have greater possibilities as to the variety of configuration they may assume, and it has been suggested (225) that the agglutinating action of viruses (80) on the red blood cells of a variety of species of animals (18) may be based on this rather than on the presence of any special constituents.

The heteroagglutination reactions obtained with the body fluids and cells of the invertebrates discussed above do not, in general, occur within a taxonomic class of animals (215). On the other hand the cross-reactions of egg waters occur with spermatozoa of closely related species. Where spermatozoa of a remotely related species are agglutinated (e.g., Nereis sperm in Arbacia egg water), the reaction is attributed (110) to a separate substance in the egg water. Whether this will be found to hold in general needs to be determined. It is quite possible that the same molecule may possess both isoagglutinating and heteroagglutinating activity. If the situation is analogous to that found for the body fluids of the lobster and the starfish, then one may expect to find a number of heteroagglutinins present in egg water. If this activity is carried by the same substance that represents the isoagglutinin then only a fraction (e.g., one-tenth if there are ten equally distributed heteroagglutinins) would possess any particular heteroagglutinating activity. Absorption of the egg water by sperm of any one remotely related reactive species would then remove only that fraction and reduce the isoagglutinating potency of the egg water relatively little.

In defining the specificity of fertilizins the above considerations should, then, be taken into account. On the basis of present evidence it appears that the isoagglutinins are dominantly species-specific.

E. Role in fertilization. 1. *Removal of fertilizin.* Various features of fertilizin (defined in particular as the isoagglutinin) led Lillie (114), Just (91) and others to consider it of essential importance in fertilization. The critical experiments concern the fertilizability of eggs that are deprived of this material. Lillie (110) reported that prolonged washing (1 to 3 days) to remove fertilizin rendered Arbacia eggs nonfertilizable. Loeb (122, 123) objected that this was due to death of the eggs during the washing period and, in Strongylocentrotus, obtained fertilization of fresh eggs deprived of detectable fertilizin by means of acid treatment. Using this method with Arbacia, Lillie (111) obtained reduction in fertilizability and noted the presence of fertilizin in suspensions of acid-treated eggs. In Strongylocentrotus, however, he (115) obtained no detectable fertilizin after such treatment and found the eggs to be fertilizable. In more recent work, following identification of fertilizin with the gelat-

inous coat of the egg, removal of this material has been found (69, 204) to impair the capacity of the eggs for fertilization. Although fertilization occurs in eggs that yield no detectable fertilizin, the jellyless eggs must be inseminated with much higher concentrations of sperm than are necessary for fertilization of the control eggs (204). It appears, then, that fertilizin serves as an aid to fertilization, its presence as the gelatinous coat enabling fertilization to be effected with smaller amounts of sperm than in its absence. While sea urchin eggs that have been deprived of their jelly layer by acid treatment, by shaking or by proteolytic enzymes, give off no further detectable fertilizin into the medium, they still react with solutions of antifertilizin from the sperm (see p. 198). This means that a layer of fertilizin remains intimately bound to the surface. The fact that jellyless eggs are still fertilizable does not, then, exclude the possibility that the presence of fertilizin may actually be essential for fertilization. This would be consistent, too, with Chamber's (15) and Just's (89) demonstration that egg fragments devoid of original surface material (cortex) are unfertilizable.

Runnström *et al.* (189) have reported experiments on so-called underripe eggs of Psammechinus miliaris in which fertilizability is evidently improved by removal of the gelatinous coat. In these 'underripe' eggs they note that the coat is greatly contracted and more solid than in the ripe eggs, due presumably to lack of water in the material of the coat. The spermatozoa cannot penetrate such a coat. Upon standing in water for some time the coat generally softens and swells and fertilization can then be effected. Removal of the contracted coat by means of acid also permits fertilization. There is, then, no direct contradiction between these experiments and those discussed above. Presumably in the contracted hydrophobic condition few, if any, combining groups of the fertilizin of the coat are available for interaction with the antifertilizin (see below) of the sperm.

In many species of marine animals the unfertilized eggs show no distinct gelatinous coat. Presumably, the surface of such eggs contains a fertilizin which would function as in the jellyless eggs of sea urchins. Aside from the case of the polychaet annelid Nereis, such eggs have not been particularly investigated in this regard. Nereis presents an interesting situation in that a gelatinous coat forms on the surface of the egg shortly after attachment of the sperm and continues to enlarge during and for a while following the slow penetration of the fertilizing spermatozoön. There is a distinct, rather tough, vitelline membrane at the surface of the unfertilized egg and the jelly exudes through this as it is converted into the fertilization membrane. Lillie (109) found that the unfertilized eggs charge the surrounding sea water with small amounts of fertilizin and that as the jelly exudes, following fertilization, large amounts are obtained. It appears likely that, in Nereis, the jelly is also composed of fertilizin which, either because of the small quantities exuded before fertilization or special properties, cannot form a gelatinous coat on the unfertilized egg. Novikoff (160) and Costello (22, 23) have discovered that treatment of unfertilized Nereis eggs with alkaline NaCl causes the jelly-precursor to swell without exuding through the vitelline membrane, thereupon rupturing the latter and going into solution itself. Costello (22) finds that such jellyless, vitelline membraneless eggs are incapable of fertilization, but if the jelly and membrane is similarly removed shortly after insemination,

normal development may ensue. These results may again be interpreted as indicating a necessity of fertilizin for fertilization, it being assumed that the fertilizin is present as the jelly precursor and as part of the vitelline membrane of the unfertilized egg. The vitelline membrane of the Nereis egg would, then, correspond to the vitelline membrane of the sea urchin egg, its location with respect to jelly material being reversed.

2. *Addition of fertilizin.* Reports that the addition of fertilizin improves the fertilizability of washed or unripe eggs (247, 53) were disputed by Lillie and Just (118). In more recent experiments by Tyler (204) the addition of solutions of fertilizin to jellyless and normal eggs of Strongylocentrotus was found to interfere with fertilization. Since agglutination is temporary in this species, the sperm being intact and active after reversal, the inhibition is not attributable simply to unavailability of free spermatozoa. Further tests confirm Lillie's (109) observation that the fertilizing power of sperm that have agglutinated and reversed is considerably reduced, the amounts of treated sperm required for fertilization being 40 to 200 times the minimum amounts of control sperm. These results do not actually conflict with those described in the preceding section which indicate a helpful action of fertilizin. It should be recalled here that fully agglutinated and reversed sperm cannot be reagglutinated. Having reacted with all the fertilizin that they are capable of binding, they would not be expected to react further with fertilizin present on the eggs. The inhibiting action of fertilizin in solution tends then to support the view that this material is important for fertilization. However, to participate in the fertilizin process it must be present on the surface of the egg. In solution it acts as a barrier to fertilization, the reaction with the sperm being completed at a distance from the egg. These considerations help explain why sea urchin eggs that have stood for a short time in unchanged sea water require more sperm for fertilization than if they are washed immediately prior to insemination. Fertilizin goes into solution as the eggs stand in sea water and effectively incapacitates some or all of the sperm depending upon the relative amounts present. As Rothschild (178) has aptly stated it, in a recent article on fertilization, a false fertilization takes place and the sperm are 'muzzled' by reaction with fertilizin in solution.

3. *Cross-fertilization.* Several investigators (109, 110, 115, 122, 123, 87, 36) have examined the question of the relation of cross-fertilization to cross-agglutination. While there are differences in detail and interpretation of their results, all agree that there is no correspondence between the degree of cross-agglutination and of cross-fertilization obtained with various species of sea urchins. Both cross-agglutination and cross-fertilization occur to a greater or less extent among most of the species of echinoids examined. As an index of cross-agglutination the relative titers of the egg waters on the various sperm is employed. For cross-fertilization it is the concentration of sperm required for the foreign eggs, in comparison with the minimum needed for the homologous eggs, that serves as a measure, it being known (115, 36) that the per cent of cross-fertilization increases with amount of sperm employed and that, in general, much more is needed for cross-fertilization than for fertilization within the species. Absence of correlation has been interpreted (123, 36) as implying lack of significance of fertilizin in fertilization. However, the reaction of the sperm with

fertilizin is undoubtedly only one of a series of reactions that may be involved in the fertilization process and its specificity would not necessarily be expected to determine the overall specificity of the process.

4. *Parthenogenesis.* Recent work on the subject of artificial activation of the egg has been reviewed by Tyler (205, 219) and R. S. Lillie (119). For the present purpose the possible role of fertilizin in the process must be considered. Moore (142, 143), Just (88) and Lillie (116) showed, in contradiction to Loeb's (123) results, that sea urchin eggs that have been optimally activated by means of butyric acid are incapable of fertilization, even after removal of their membranes. Since, after butyric acid treatment, fertilizin can no longer be obtained from the eggs Lillie and Just considered lack of its further production to be responsible for the failure of superposition of fertilization on parthenogenesis. However, since fertilizin has now been identified as a component of the gelatinous coat of the egg and since this coat is dissolved by butyric acid treatment (110, p. 559), their interpretation no longer appears warranted. A similar interpretation of the block to polyspermy, or failure of fertilized eggs to refertilize, was based on the view that fertilizin was continuously produced by the unfertilized egg, but production ceased after fertilization. However, Lillie (110, p. 553) noted that demonstration of cessation of production depended upon removal of the gelatinous coat of the egg which he found to contain fertilizin in high concentration. This feature of the experiment is evidently responsible for what now appears to be a misinterpretation of the facts. In this connection it may be mentioned that refertilization of fertilized eggs has been obtained under special conditions (228). There does not, then, appear to be much justification, as yet, for assigning to fertilizin a rôle in artificial activation or in the establishment of the block to polyspermy. Further investigation along this line would be desirable.

ANTIFERTILIZIN FROM SPERM

The substance of the sperm with which fertilizin combines has been termed antifertilizin (110). It was first extracted from sperm by Frank (44) and Tyler (200) by different methods and is reported to have various effects on eggs and on spermatozoa.

A. EFFECTS. 1. *Neutralization of fertilizin.* Extracts of sperm of sea urchins and mollusks prepared by brief heating in sea water (44) or by freezing and thawing (200) are found to be capable of neutralizing the agglutinating action of fertilizin on homologous sperm. In the supernatant obtained by centrifugation of 'dry' or concentrated sperm suspensions this agglutinin-neutralizing property has been reported to be present by Southwick (194) and Hartmann *et al.* (69), absent by Frank (44) and Hayashi (71) and weak by Tyler and O'Melveny (226). Hartmann *et al.* (69) also reported that both the supernatant and the extracts neutralize the sperm-activating component of egg water in Arbacia pustulosa, while Frank (44) found no such action of sperm extracts in Arbacia punctulata. Runnström *et al.* (189, 184, 185) and Vasseur and Hagström (230) likewise obtained negative results with the supernatant of sperm of Psammechinus miliaris and Echinocardium cordatum.

The antifertilizin is evidently rather firmly bound to the sperm; however, it does, in our experience, slowly dissolve as the sperm age in sea water, and slight acidification (pH 4–6) of fresh suspensions yields substantial amounts without killing the

sperm. Extracts prepared by disruption of the cells neutralize more fertilizin than is absorbed by an equivalent amount of live sperm. Evidently, not all of the antifertilizin carried by the sperm is available for immediate action.

2. *Agglutination of eggs.* Another effect of sea urchin, sperm-extracts consists in agglutination of homologous eggs (246, 44, 203, 226, 189). Microscopic examination shows that a precipitate forms on the surface of the gelatinous coat, outlining the latter sharply. The reaction resembles the so-called Neufeld reaction obtained with encapsulated micro-organisms, such as pneumococci, in the presence of specific antisera. Agglutination of the eggs and formation of the precipitation membrane are quite evidently two aspects of the same reaction. It is also clear that the agglutinating substance in the sperm-extract reacts with the gelatinous coat of the egg, and various tests (44, 203, 226) identify the egg-agglutinin with the agent (antifertilizin) that neutralizes the sperm-agglutinin (fertilizin). The term antifertilizin may then be used for the substance having these two effects. Another manifestation of the same reaction is the formation of a precipitate when solutions of fertilizin and antifertilizin are mixed under appropriate conditions (44, 203). Eggs that have been deprived of their gelatinous coat by shaking or by acid treatment are also agglutinated by solutions of antifertilizin and the latter is absorbable by such eggs. There is, then, present on the surface of such eggs a layer of fertilizin that is more intimately bound than is the rest of the gelatinous coat.

3. *Dissolution of the gelatinous coat.* According to Hartmann *et al.* (69), sperm-extracts of Arbacia pustulosa also have the property of dissolving the gelatinous coat of the egg. Tyler and O'Melveny (226) have noted a similar disappearance of the gelatinous coat of eggs of Strongylocentrotus purpuratus and Lytechinus anamesus but do not interpret this as dissolution. If the action of a strong sperm-extract is followed under the microscope a precipitation membrane is first observed to form on the surface of the gelatinous coat. The latter then contracts and the precipitation membrane becomes increasingly more distinct until it reaches the surface of the egg itself. It is not then readily distinguished from the surface of the egg. However, in the process some of the eggs may be freed of their gelatinous coat, particularly if they are shaken. Long after the precipitation membrane has shrunk to the surface and apparently disappeared on the intact egg it can be seen on the isolated jelly hulls as a distinct heavy membrane forming a hollow sphere that contracts to less than one-half of the diameter of the egg proper. It appears, then, that the material of the gelatinous coat becomes incorporated in the precipitation membrane, where it occupies considerably less volume than in the original gel form. There is, as yet, no evident necessity for the assumption of a jelly-dissolving agent in the sperm-extracts unless the recent findings of Monroy and Ruffo (140, 180), discussed in connection with hyaluronidase, refer to something other than the above sequence of events, and receive confirmation.

4. *Paralysis of the spermatozoa.* In several species of sea urchins the supernatant fluid (seminal fluid) obtained by centrifugation of 'dry' or concentrated sperm-suspension has been found to have an immobilizing action on the homologous sperm. Positive results in such experiments have been presented by Southwick (194) on Echinometra subangularis, by Hartmann, Schartau and Wallenfels (69) on Arbacia pustulosa

and by Vasseur and Hagström (230) on Psammechinus miliaris. On the other hand, in Echinocardium cordatum, Runnström, Tiselius and Lindvall (188) find no immobilizing action of seminal fluid and, in Arbacia punctulata, Hayashi (71, 72) finds that it actually maintains the motility and respiratory activity of the sperm. Hartmann *et al.* (69) found their sperm-immobilizing agent to be soluble in methanol. In partial accord with this, Runnström and Lindvall (183) obtain inhibition of motility with a methanol extract of E. cordatum, but not of P. miliaris, sperm. This methanol extract is also found to have a certain lytic action on the egg surface which will be discussed below. Hayashi's (71, 72) seminal fluid factor is found to be nondialyzable and precipitable by ammonium sulphate.

In the work of Runnström *et al.* (183, 188), Vasseur and Hagström (230) and Hayashi (71, 72) possible effects of CO_2 tension and pH were taken into account. It would appear then, that different species differ considerably in regard to the action of seminal fluid and sperm extracts on sperm. No general conclusions can, therefore, be made as yet concerning the relation of such action to antifertilizin activity.

B. CHEMICAL PROPERTIES. Various workers have examined the chemical properties of antifertilizin (as defined by agglutinating action on eggs, formation of precipitation membrane or neutralization of the sperm-agglutinin). Frank (44) found the active agent in Arbacia punctulata to be nondialyzable, relatively heat stable, insoluble in alcohol and ether and nonprecipitable by ammonium sulphate. His preparations gave negative results in the common tests for proteins and also failed to reduce Benedict's solution or Nylander's reagent. With solutions of Strongylocentrotus and Megathura antifertilizin, that were evidently stronger, Tyler (200, 203) obtained positive protein reactions, precipitation with ammonium sulphate and inactivation by proteolytic enzymes. In Arbacia pustulosa (69) and in E. cordatum (189) the active agent is also found to be insoluble in methanol, nondialyzable and relatively heat stable. Upon ultracentrifugation of a dialyzed sperm extract Runnström, Tiselius and Vasseur (189) find a main component with a molecular weight under 19,000 and no appreciable amounts of any larger molecules. Upon electrophoresis they find only one component and this behaves as an acid down to pH 4. Tyler and O'Melveny (226, 216) showed that antifertilizin of Strongylocentrotus and Lytechinus is extractable in acidified sea water (ca. pH 3.5). Dialysis, ammonium sulphate precipitation and isoelectric precipitation (pH 3.0 in the absence of salts) yields an electrophoretically homogenous preparation (216) whose single component shows mobilities that correspond closely with those reported by Runnström *et al.* Purified antifertilizin preparations of Lytechinus are found to contain about 16 per cent nitrogen. Solutions containing 0.2 to 0.3 mgm. N/ml. give titers (maximum dilution giving visible precipitation membrane when tested with an equal volume of a suspension of ca. 10,000 eggs per ml.) of 128 to 256.

The antifertilizin preparations are found (226, 216, 183) to be antigenic in rabbits, the antibodies agglutinating the intact sperm as well as precipitating with original solutions. This is consistent with both its protein nature and location on the surface of the spermatozoa. Electron microscope examination of spermatozoa from which antifertilizin has been extracted (pH 3.5 to 2.8) show (216) no effect on the acrosome, midpiece or tail, while the intervening region of the head (which is normally elongate)

becomes spherical, increasingly swollen and less dense. The antifertilizin is evidently present on this region of the surface of the head.

C. SPECIFICITY. Since antifertilizin reacts with fertilizin its specificity may be expected to parallel that of fertilizin. However, different tests, corresponding to different manifestations of the interaction, may give different results. Thus, if one of these agents is effectively 'univalent' with respect to the other in homologous or cross-reactions, the results obtained by direct agglutination tests would differ from those obtained by neutralization tests. Comparisons on this basis have not as yet been made, but several workers have obtained data on cross-reactions of antifertilizin. Frank (44) showed that the egg-agglutinin was not obtainable from tissues or fluids of Arbacia other than the sperm (see below for an antifertilizin from eggs) and that it acted only on the eggs. Reciprocal cross-reactions were obtained with another echinoid, Echinarachinus. Cross-reactions among the echinoids, tested by egg-agglutination, or in some cases by inhibition of sperm-agglutination, have also been reported by Tyler (203), Hartmann *et al.* (69) and Runnström *et al.* (189). In addition, however, reactions with material of remotely related species have been reported. Thus Frank (44) obtained agglutination of Arbacia eggs with sperm extract of the polychaet annelid Nereis, but not the reciprocal. Runnström *et al.* (189, 184) obtained agglutination of sea urchin eggs with extracts or salmon and bull sperm and with serum of a shark and a bony fish. These evidently represent heteroagglutination reactions of the type discussed above (see p. 189). It may be recalled that egg water and blood of Arbacia agglutinates Nereis sperm, and that the seminal fluid of various species of animals has agglutinating action on sperm and blood cells (and probably eggs) of different assemblages of whole groups of animals (224, 225, 227, 215). To what extent such heteroagglutinating action may be the property of substances distinct from the isoagglutinin or the property of the same molecules involved in isoagglutination needs to be determined. It is clear, however, that the heteroagglutinating action of the materials obtained from sperm is a property that such material has in common with substances of the body fluid (and probably other tissues) of the body. Thus in defining the specificity of antifertilizin (or of fertilizin) cross-reactions that are also given by body fluids or extracts of other tissues may be ruled out. On this basis it appears that the fertilizin-antifertilizin reaction is dominantly species-specific.

D. ROLE IN FERTILIZATION. 1. *Removal or blocking of antifertilizin.* To test the significance of antifertilizin in fertilization it would be desirable to deprive the spermatozoön of this agent. Complete removal without destruction of the sperm has not proved feasible. However, when antifertilizin of Strongylocentrotus and Lytechinus is partially extracted by means of slightly acidified sea water or warm sea water, the spermatozoa are found to remain viable and motile (Tyler and O'Melveny, 226). Tested on eggs, such extracted spermatozoa showed a very much reduced capacity (in terms of amount required) for fertilization, roughly in proportion to the amount of antifertilizin removed. At the same time their rate of oxygen consumption was found to be considerably less affected. Thus, in different tests, sperm having 80 per cent of the control respiratory activity showed between 3 and 25 per cent of the control fertilizing capacity and when respiratory rate was reduced to 25 per cent, fertilizing

capacity was found to be less than one percent. While other effects of the treatment are not ruled out, the experiments indicate that deficiency in antifertilizin impairs fertilizing capacity.

Another method of eliminating antifertilizin without physical damage to the sperm consists in neutralizing it by means of a specifically reacting agent. This has been done by reaction with fertilizin, as described above (p. 000), with the result that the sperm become relatively nonfertilizing. It has also been attempted by means of antibodies produced in rabbits by injection of purified antifertilizin (Tyler and O'Melvény, 226; Tyler, 216). Since the spermatozoa are agglutinated by the antiserum any interference with fertilization could be attributed to the locking up of the sperm and the experiment would supply no information concerning the possible effect of neutralizing the antifertilizin. To overcome this difficulty, use was made of 'univalent' antibodies prepared by photo-oxidation of the ordinary antiserum. After treatment with such 'univalent' antibodies the spermatozoa were found to be of normal appearance and motile. When tested on homologous eggs in experiments with Lytechinus and with the gephyrean worm Urechis, the treated sperm showed a considerable reduction in fertilizing power, roughly in proportion to the titer of antibodies employed for treatment. The heterologous antibodies were found to be ineffective. This experiment further supports the view that antifertilizin is involved in the fertilization process. It does not, however, necessarily mean that the antibodies bodies combine with the same specific groups on the antifertilizin molecule that are involved in the interaction of the latter with fertilizin. The antibodies might very well be directed against other specific groups, and, in combining, physically block those concerned in the reaction with fertilizin.

2. *Some immunological analogies.* In the field of immunology analogous experiments are employed in attempting to identify the factor involved in virulence of pathogenic organisms. Thus, for example, it has been shown that antibodies directed against the polysaccharide coat of pneumococcus will reduce the infective power of the organism. A further analogy is provided by experiments on bacteriophage treated with a specifically reacting substance of the susceptible bacterium or with antisera. Thus Levine and Frisch (105) and Gough and Burnet (58) showed that phage can be specifically inactivated by a substance derived from the coat of the susceptible organism. The infective power of phage on the bacteria can also be impaired by means of antiphage immune sera, the inactivation following a percentage law (30, 8). According to Burnet (7, 8) the antibodies combine with specific groups other than those by which the phage particles react with the surface of the bacterium but may spatially block some of the latter and thus interfere with adsorption of phage to the bacterial surface. The action of specific bacterial substance and of specific antibody on phage resembles then the action of fertilizin and of specific antibody on sperm. There is also evidence (see Delbrück, 28) that only a single phage particle (perhaps the first to attach to the surface) actually enters the bacterial cell and multiplies. This would imply a further analogy with the phenomenon of monospermy or establishment of the block to polyspermy in fertilization. Of course, the outcome of phage action in producing lysis of the bacterial cell is quite different from the development that normally ensues following fertilization, but it may be mentioned in passing

that Lindahl (120) has reported cytolysis of specially treated eggs as a result of fertilization. Some bacteriophage particles seem to be provided with a tail (see 126), but this slight structural resemblance may be taken less seriously than the above analogies.

3. *Sperm-immunity and sterility in mammals.* Numerous investigators have examined the possibility of inducing sterility in various animals, including humans, by means of antibodies against homologous or heterologous sperm or their extracts. A brief review of the subject has been previously presented (206). Although there have been many conflicting reports, the evidence as a whole is to the effect that sterility is not induced by such immunization of females even when antibodies are found to be present in the serum of the animals at the time of mating. In one type of experiment a temporary sterility is evidently obtained. This consists in the introduction of the antispermatozoal serum into the vaginas of rabbits followed by matings within one to 30 hours (Parsons and Hyde, 165). The experiment is essentially a direct treatment of the sperm with antibodies such as reported above with invertebrates and it is not surprising that the results are similar. The negative results in other types of experiments involving active or passive immunization of the female may be attributed to unavailability of the antibodies, insufficient concentration relative to the sperm or other factors.

4. *Addition of antifertilizin.* Treatment of sea urchin eggs with antifertilizin or insemination in the presence of antifertilizin has been found (44, 69, 203, 226) to inhibit fertilization. It has also been reported by Frank (44) that treatment of the sperm with antifertilizin impairs their fertilizing capacity, but this has been denied by Hartmann *et al.* (69) and by Tyler and O'Melveny (226). Reaction of antifertilizin with the egg results in the formation of a precipitation membrane as described above (p. 194). In dilute solutions this membrane may be incomplete and it has been noted, in another connection (203), that such eggs may be fertilized. More complete interaction with antifertilizin renders the eggs nonfertilizable. Eggs deprived of their gelatinous coat are also found to lose their fertilizability upon treatment with antifertilizin (44). This may be interpreted as support for the view mentioned above (p. 191) that a layer of fertilizin remains on the surface of the egg after removal of the coat by the usual methods. In any event it appears that antifertilizin in solution acts as a block to fertilization, its interaction with fertilizin of the egg evidently preventing interaction of the latter with the antifertilizin present on the sperm. As in the case of the fertilizin discussed above (p. 191), the substance must remain on the surface of the cell in order to function in the fertilization process.

5. *Alexin (complement) and the fertilizin-antifertilizin reaction.* The interaction of fertilizin and antifertilizin has been considered to be analogous to that of antigen and antibody. An interesting feature of antigen-antibody reactions is their ability to bind alexin (usually called complement—a heat labile, relatively nonspecific material present in the serum of vertebrate animals, the action of which is ordinarily manifested by the lysis of erythrocytes, or other cells, when reacting with specific antibody in its presence). Ability to bind alexin is not, by any means, characteristic of all antigen-antibody reactions. There are a great many exceptions, such as in toxin-antitoxin reactions (see Osborn, 161), reaction of pneumococcal capsular polysac-

charide with its specific antibody produced in horses (251) or in humans (197), and most cases of the reactions of the normal blood-group isoantibodies (see 241). In many cases, too, antigen or antibody alone may combine with alexin (termed anticomplementary action) to such an extent that no decision can be made as to whether or not alexin is fixed by their interaction. It is of interest, then, to learn how the fertilizin-antifertilizin reaction behaves in this regard. This was examined by Tyler (208) with fertilizin and antifertilizin of Strongylocentrotus and alexin of guinea-pig serum. It was found that the fertilizin alone bound alexin. Upon reaction with antifertilizin a rather novel situation was discovered. The antifertilizin destroyed the ability of fertilizin to combine with alexin, or, if the alexin was first bound by fertilizin, addition of antifertilizin released it. This alexin-release reaction could in fact serve as a measure of the fertilizin-antifertilizin reaction, just as, conversely, alexin-fixation is used to follow antigen-antibody reactions. 'Univalent' fertilizin by itself was also found to bind alexin which could be released by antifertilizin. Alexin is known to be composed of two heat-labile and two relatively heat-stable components (see 34, 76). It is with the relatively heat-stable fourth component (C′4), which is found in a muco-euglobulin fraction of serum, that the fertilizin was found to combine. Since antifertilizin and C′4 compete in the interaction with fertilizin, it might be expected that antifertilizin could substitute for C′4 in the hemolytic action of alexin. Tests of this possibility gave negative results. This means that the antifertilizin is quite different from C′4 in those properties that enable the latter to act with the other three components and the sensitized cells in the hemolytic system. The binding of alexin by fertilizin is also manifested by agglutination of the sea urchin eggs and formation of the precipitation membrane in guinea pig serum. This may, then, be simply another aspect of the heteroagglutination type of reaction above (p. 190) and it seems reasonable to conclude that the specific groups by which fertilizin combines with antifertilizin are different from those involved in union with C′4, the release phenomenon being the result of spatial interference due to proximity of the different groups.

6. *A hypothetical scheme for attachment of the sperm.* On the basis of the present evidence fertilizin and antifertilizin appear to be concerned primarily with attachment of the spermatozoön to the egg. The sperm-activating agent associated with fertilizin may be helpful in speeding up encounter of the gametes and in stimulating fatigued sperm to an extra burst of energy for entrance into the gelatinous coat, but there is little to substantiate this. Since the gelatinous coat is composed primarily of fertilizin, the question may be raised as to whether the spermatozoön reaches the egg surface proper (vitelline membrane) before it has reacted completely. Experimental evidence concerning this is lacking, but to provide a basis for discussion the following picture of the mechanism of attachment of the sperm may be suggested. The fertilizin comprising the gel micelles of the coat of the egg would have relatively few combining groups available for reaction with the sperm. Additional combining groups would be available on the egg surface proper. A small amount of fertilizin present in solution in the interstices of the gel would react with the sperm or with any antifertilizin that may dissolve off it. The effective initial reaction would, however, be between the active groups available on the micelles of the gel and on the surface of the sperm. These groups are probably absent from the acrosome and midpiece of

the sperm (see p. 195). If the complementary fertilizin groups are radially arranged in the gel and absent from its outermost surface, then on contact with the latter the spermatozoön would be held in a position normal to the egg surface. Since interaction of fertilizin and antifertilizin results in precipitation (presumably because the specific combining groups are also the ones that render these substances hydrophilic and the interaction destroys their affinity for water) the micelles would also tend to precipitate. Since it is anchored to the surface of the cell, precipitation of a micelle would be manifested by its contraction. The spermatozoön would thus be brought into contact with more deeply located combining groups and cause further contraction of micelles until the surface of the egg is reached. Radial arrangement of the available groups in the gel would keep the spermatozoön oriented normal to the egg surface, where further interaction of the fertilizin on the vitelline membrane with the laterally disposed antifertilizin on the head of the sperm would result in more intimate contact and could even account for the depression of the membrane that is seen at the point of attachment. To speculate further, reaction with the fertilizin of the vitelline membrane might involve a weakening of the latter, so that the egg substance tends to protrude in the form of the entrance cone which engulfs the sperm and is then partially (e.g., extrusion cones) or completely retracted into the egg by purse-string closure of the plasma membrane, as the vitelline membrane is converted into the fertilization membrane. To account for the block to polyspermy one might assume a rapid, chain-like reorientation of the molecules comprising the vitelline membrane in response to the first sperm to attach so that the supernumeraries cannot attach as firmly. One might also assume that a lytic agent such as that described below (p. 206) is involved in penetration and block to polyspermy.

Detailed support for the above kind of picture of approach and attachment of the spermatozoön will not be attempted here but a few observations pertaining thereto may be presented. The sea urchin spermatozoön does not swim very actively as it passes through the coat of the egg; rather the tail becomes progressively motionless as it enters the jelly. The intimate attachment to, and depression of, the vitelline membrane may not, then, be attributed to mechanical activity of the sperm. The first observations of fertilization, made by Fol (41, 42) on the starfish egg, showed this very strikingly along with the formation of a curious filament which Chambers (16, 17) and Hörstadius (81) have carefully studied. When the starfish spermatozoön strikes the surface of the coat of the egg it becomes motionless and a filament appears extending from the head of the sperm directly to the vitelline membrane. The filament then contracts dragging the sperm with it until it reaches the surface of the egg. A single egg may show a large number of such filaments extending to many of the spermatozoa on the surface of the jelly coat and their contraction brings many of the supernumerary sperm into contact with vitelline membrane where their entry is evidently blocked. According to Hörstadius the filament has the form of a hollow cylinder. I should interpret the formation and contraction of the filament to be due to precipitation of the fertilizin micelles of the gel by interaction with antifertilizin of the sperm as described above. Runnström (182) has examined starfish sperm extracts for the presence of antifertilizin and obtained no precipitation membranes on the surface of the coat of the egg. However, since the presence of fertilizin in solu-

tions of starfish egg jelly has been convincingly demonstrated (133), antifertilizin must evidently be present on the sperm. Runnström's failure to obtain precipitation membranes with sperm extracts may, then, be attributed to other factors, such as occurrence of antifertilizin in 'univalent' (nonprecipitating) form in the extracts or condition of the egg coat (e.g., few combining groups available on the surface) at the time of testing. The above account also implies that the spermatozoön must approach radially. This is consistent with most observations (see Morgan, 144) showing failure of obliquely approaching sperm to attach. In connection with the postulated radial structure of the gelatinous coat of the sea urchin egg, Runnström *et al.* (187) and Monné (139) observed no birefringence under normal conditions. A strong birefringence appeared, however, upon addition of antifertilizin or upon staining with acridine-orange. This was found to be negative in the radial direction with the former agent and positive with the latter. Monné points out that the isotropy of the normal coat may be attributed to irregular or to regular structure, the latter being obscured by compensating positive and negative birefringencies of its constituents or by hydration of a birefringent substance present in small amount. The ready appearance of double refraction upon treatment may be taken to favor the latter view, but further work will be necessary to decide the question.

In attempting at the present time to present a picture, such as the above, of the manner in which fertilizin and antifertilizin may act in effecting attachment of the sperm, one runs the risk of having the evident weaknesses of a particular scheme confused with the evidence that such substances are definitely concerned in the processes under consideration. However, I feel sure that most workers in the field will not fail to make the distinction. The great extent to which the present picture is obscure and hypothetical is a measure of the lack of specific information. It may be hoped that this attempt to visualize a mechanism will help stimulate experimentation designed to discover the true mode of action of the substances involved in fertilization. The problem of sperm attachment may seem of rather minor, special importance to some biologists. It should, then, be remarked that it is part of the general problem of the adherence of the cells of the various tissues and organs to form a differentiated multicellular organism and that it has seemed reasonable to assume (see Tyler, 220; Weiss, 239) that the same kind of factors are in operation.

ANTIFERTILIZIN FROM EGGS

A. Effects. Lillie (110) presented evidence to show that the interior of the egg of Arbacia contained a substance (that he also termed antifertilizin) capable of inactivating fertilizin. Tyler (203) confirmed this with extracts of frozen and thawed Strongylocentrotus eggs that had been deprived of their gelatinous coat and showed that the extracts would agglutinate intact eggs and cause the formation of a precipitation membrane on the surface of the gelatinous coat. [For the record it may be mentioned that Runnström (181) had earlier observed precipitation of the gelatinous coat of Arbacia eggs by means of an extract of the eggs but did not attempt to relate this to fertilizin-antifertilizin reactions.] No particular action of the extracts on the sperm has been noted. The active agent in the egg extract behaves, then, in a manner similar to the antifertilizin obtained from sperm and it is perhaps justifiable to

use the same term for both. As in the case of the antifertilizin from sperm, absorption of the extracts with eggs or treatment with solutions of fertilizin rapidly neutralizes the antifertilizin. When intact fresh eggs are extracted by freezing and thawing in sea water, no antifertilizin is generally obtained in the solution. This is evidently due to its neutralization by the fertilizin of the gelatinous coat. From freshly shed eggs, that are provided with a large coat, the relative amounts of fertilizin and antifertilizin extractable is of the order of ten to one, as tested by reciprocal neutralization. The occurrence of antifertilizin within the cell along with fertilizin on the surface has interesting implications that will be discussed further below.

B. Chemical and antigenic properties. Some information concerning the chemical nature of the antifertilizin of eggs has been obtained by Tyler (203, 221). For extraction it is of importance first to remove the coat of the egg, since this would otherwise neutralize much or all of the antifertilizin. Freezing and thawing of the jellyless eggs of Strongylocentrotus yields a coagulum of insoluble egg material along with a yellowish colloidal solution containing the antifertilizin. The active agent is nondialyzable and precipitates in ammonium sulphate or, in the absence of salts, at pH 4 to 4.5. It is insoluble in alcohol and inactivated by heat and by proteolytic enzymes. While there is resemblance to the antifertilizin of sperm in these few properties, much further information will be needed before any definite conclusions can be drawn concerning the extent of similarity or dissimilarity. The present evidence indicates that the material is a protein.

The solutions of antifertilizin from eggs are found to be active antigens. The antiserum, obtained by immunization of rabbits with such solutions, neutralizes and precipitates the antifertilizin but does not agglutinate the intact eggs. The latter effect is understandable since the antifertilizin is a subsurface antigen. On the other hand, the antiserum does agglutinate homologous sperm. This means then that the antifertilizin from eggs is antigenically similar to that from sperm. It does not, however, necessarily imply any overall chemical similarity.

C. Role in fertilization. Direct tests of the function of the antifertilizin of eggs by removal or neutralization experiments are not readily feasible. Treatment of eggs with the solutions is found (203) to interfere with fertilization, as in the case of treatment with the antifertilizin of sperm.

Lillie (114) proposed that the antifertilizin of eggs is involved in the establishment of the block to polyspermy. He pictured it as reacting with fertilizin immediately upon fertilization, thereby rendering the latter unavailable for reaction with additional spermatozoa. Since apparent secretion of fertilizin by unfertilized eggs and its cessation upon fertilization is now attributable to dissolution of the gelatinous coat, the proposal in its original form seems no longer tenable. It is conceivable that the antifertilizin might act in some such manner by interaction with a layer of fertilizin on the vitelline membrane of the egg, but experimental evidence concerning this is completely lacking.

Concerning the specificity of the antifertilizin from eggs, present evidence shows cross-reaction between the related, cross-fertilizing species of echinoids, S. purpuratus, S. franciscanus, Lytechinus pictus and Dendraster excentricus.

D. NATURAL AUTO-ANTIBODIES. 1. *Terminology.* In considering the fertilizin-antifertilizin reaction to be analogous to that of antigen with antibody, one may ask which represents antigen and which antibody. With the usual immune bodies the answer is relatively simple. The antibody is the substance that is produced as a result of injection of the antigen and is ordinarily found in a protein component of the serum of the immunized animal. The antigen is the inducing agent, which may be a protein or polysaccharide in solution or as part of a cell and which will react with the antibody to give precipitation, agglutination, alexin fixation, etc., depending upon the conditions of the reaction. The term is also applied to substances (simple and complete haptens) which by themselves are incapable of inducing antibody-formation but can react with the specific antibodies. When normally occurring substances, such as those involved in the blood group reactions, are considered, the definitions must be broadened somewhat. In this case the agglutinins are present in the non-immunized animal, having been formed as a result of gene-controlled reactions. They are generally termed antibodies by immunologists because of their presence in the serum, but such designation immediately conflicts with that part of the definition of antibody as something produced as a result of the introduction of a foreign substance into the organism. Likewise, designation of the so-called agglutinogens of the erythrocytes as antigens broadens the definition of that term. There are also cases where two substances occurring normally in the sera of different animals interact, as for example in the inhibition of blood group reactions (241). The designations in such cases would depend upon the direction in which the test is set up. This is the type of situation involved in attempting to use these terms for fertilizin and antifertilizin. Thus fertilizin can act as an agglutinin for sperm and might therefore be termed an antibody. But antifertilizin can act as an agglutinin for the eggs and should likewise be termed an antibody. Rather than abuse the terminology to that extent it is preferable to refer to such substances as complementary substances (complementary proteins, etc., further designation depending upon their chemical nature) of which the more familiar antigens and antibodies form a class whose manner of interaction is fundamentally the same. Professor Sterling Emerson, of this institution, has suggested (not too seriously) alleloplastology in place of immunology to designate the study of such reactions which are involved in problems of biological specificity. The reactants might then be termed alleloplasts. Some such general terminology would have definite use. The purpose of mentioning Emerson's suggestion here is to encourage consideration of the terminology. In the meantime substances like fertilizin and antifertilizin may be designated mutually complementary substances whose interaction occurs in the manner of antigen and antibody.

2. *Mutually complementary substances in cells.* The finding of antifertilizin within the egg means, then, that a single cell contains a pair of components, capable of interacting with one another in antigen-antibody manner. The implications of this and related findings in immunological and general biological problems have been presented in previous publications (Tyler, 203, 206, 217, 220) and only a brief summary of some of the points will be given here.

It is of importance first to know whether or not the situation is general for all kinds of cells. In immunological literature there are a number of reports of agglutination

or lysis of cells by means of extracts of the same cells. The present author, too, has been able to extract such auto-agglutinins from various bacteria and red blood cells, but not consistently. One of the difficulties evidently involved in such attempts is the probable occurrence of interaction and precipitation of the complementary substances in processes of extraction that involved destruction of the whole cell. If surface substance be present in excess it would bind the complementary subsurface substance and the latter would not be obtained in the ordinary extracts. Since the complementary substances may combine in multiple proportions and since one or both may be 'univalent' there is further difficulty in extraction and identification. In attempting to extract a complementary subsurface material it is evidently essential first to remove the surface substance. While this is readily feasible in the case of the sea-urchin egg, it is more more difficult in other kinds of cells. These factors may account for the above-mentioned lack of consistent results and more significance may, then, be attached to cases of active extracts.

There is no reason to expect complementary substances to occur simply in pairs, so in the present state of our ignorance we may conceive of cells as constructed of a number of complementary substances. For purpose of emphasis I have termed this the auto-antibody concept. Since the complementary substances are capable of combination, it is pertinent to ask how they may exist together in the cell. The tentative answer is that they are actually in combination in the regions in which they adjoin and by their union form the various membranes on and within cells. The manner of formation of the complementary structures will be considered below.

In a multicellular organism it may be expected that some of the surface material of the cells of various tissues would also be present in the body fluids. On this basis the demonstration by Kidd and Friedewald (92) that normal rabbit serum reacts, in antibody manner, with a sedimentable constituent of extracts of various organs of the same animal is consistent with the auto-antibody concept. A more familiar auto-antibody is the so-called Wassermann reagin found in syphilitic serum. Similar examples are known in other diseases, such as infectious mononucleosis, yellow fever, acute hepatitis, malaria and virus pneumonia. The reagin reacts with a lipoid substance that is extractable from the tissues of the same animal. Since it appears after infection it is assumed to be an immunologically induced antibody. The usual interpretations assume either that the lipoid tissue constituent combines with some protein of the infecting organism to form a complete antigen capable of inducing the formation of antibodies directed against the lipoid or that the pathogen possesses an antigen that is serologically similar to the tissue lipoid. A third interpretation is provided by the auto-antibody concept. It assumes that, in the tissue destruction occasioned by the pathogen, lipoid and complementary protein are liberated and that the smaller lipoid molecules are more rapidly eliminated from the body, leaving the complementary protein present in excess in the circulation and capable of reacting in vitro with added lipoid. Agents that tend to increase the concentration of lipoid in the blood would presumably render the serum nonreactive and this may explain why alcohol imbibition results in false negative Wassermann reactions. It should, on this basis, also be possible to cause false positive reactions without infection, and in this connection it may be noted that donors for blood banks have often been found

to give false positive reactions after repeated bleedings. Presumably the drain on the tissue cells in replacing the serum proteins results in liberation of some protein that is complementary to the tissue lipoid. While real evidence is as yet lacking, these examples perhaps suffice to show that the question is subject to experimental attack.

3. *An auto-antivenin.* A practically, as well as theoretically, important property of immune antibodies induced against pathogenic organisms, toxins or venoms, is their ability to act as protective agents. It is of interest, then, to learn whether the so-called auto-antibodies might serve in that manner. Experiments by the author with extracts of pneumococci have not, as yet, yielded definite results one way or the other. However, in other experiments with a venomous reptile called the Gila monster, material capable of neutralizing the venom of the same animal as well as of other individuals has been obtained (217). This material was found in the serum and in extracts of the liver of the animal, but not in extracts of the venom glands where it was first sought. The latter point would be difficult to reconcile with the auto-antibody concept if the venom gland is the site of synthesis of the venom. However, this is not known to be the case and the evidence (see 125) indicates that it may be produced elsewhere and transported in bound form to the gland where it is released. The antivenin in the blood may, then, represent complementary substance left behind when the venom is taken up by the gland, both substances presumably coming from the liver. Aside from the precise interpretation the finding of antivenin in the animal is favorable to the view that naturally occurring complementary substances may act as protective agents.

There are many examples, that may be cited from the immunological literature, of the destruction of cells by means of substances derived from the same kind of cell. Thus certain antibacterial proteins such as lysozyme and diplococcin are derivable from the same species of organism on which they act (135, 162). Certain autolytic enzymes perhaps belong in this category, although in most instances they do not attack the intact cell (see 31). Bacteriophage may be regarded as an example of this type since it attacks the cell from which it is produced and is known to react in antibody-like manner with a surface constituent of the cell (see 24). In fact it seems just as reasonable to regard the virus as being originally a gene-like cellular component as to consider it a distinct parasitic living entity. The facts of spontaneous lysogenicity would perhaps be better understood on that basis than on the basis of phage carriers.

4. *Antibody formation.* The antigen-antibody analogy leads to the view that the complementary substances of cells are formed by the same kind of processes as are involved in antibody formation. The exact mechanism of antibody formation is not known, but there is now wide acceptance of the general theory proposed by Breinl and Haurowitz (5), Alexander (2) and Mudd (157) which has been extended and experimentally supported by Pauling and Campbell (166–169). Briefly, this theory involves incorporation of the foreign antigen into the site of synthesis of serum globulin, where it so influences the construction of the globulin that various regions of the latter become structurally complementary to specific regions of the antigen. In this general theory no statement is made as to the structure of the globulin in absence of foreign antigen. On the basis of the auto-antibody concept I have proposed that the

normal globulin is similarly complementary to the substance comprising the normal site of synthesis. The formation of serum globulin may be regarded as an expression of the normal process by which the cells form various macromolecular constituents including those involved in its own growth. These constituents, then, would be complementary to the substance or substances comprising the site of synthesis, i.e., synthesis of cellular constituents would occur in the same manner as antibodies. Many pairs of such mutually complementary substances should be extractable from cells plus the surrounding fluids. For the formation of self-duplicating bodies (e.g., genes) of cells, it would be necessary to assume either direct mirror-imaging of identical materials as Pauling and Delbrück (170) have done or formation of an intermediate template which Emerson (37) lists as an alternative.

The specific structure of many constituents of cells is now known to be genetically determined. This is particularly well exemplified in the work on the antigens of the blood cells (see 83). It seems reasonable to conclude, then, that the configuration of various specific structures of the macromolecular constituents of cells are the same or the complement of those on the genes.

The bearing of the auto-antibody concept on problems of differentiation involves further speculation which has previously been sufficiently indulged in (203, 206, 217, 220) and which has received some possible experimental support (Weiss, 239).

LYTIC AGENTS FROM SPERM

A. Effects. 1. *Egg-membrane lysis.* The unfertilized eggs of many species of animals among the mollusks, fish and amphibia normally possess viscous coats and a rather tough membrane which elevates from the surface when the eggs are shed into their aqueous medium. To effect fertilization the sperm must penetrate these barriers. Several early workers have postulated the presence of a lytic agent on the spermatozoön to effect penetration. Hibbard (70) and Wintrebert (244, 245) observed that extracts or macerated suspensions of spermatozoa of the amphibian Discoglossus would break down one of the coats of eggs of that species. Tyler (200) found that sea-water extracts of frozen and thawed sperm of two mollusks, the keyhole limpet Megathura crenulata and the abalone Haliotis cracherodii, were able to dissolve the egg membrane in the respective species. Von Medem (232) confirmed this with other species of keyhole limpet and abalone. Cross-reactions were not obtained with these mollusks, but more closely related forms have not been tested.

The process of dissolution of the membrane can be readily followed in the eggs of mollusks. The membrane becomes thin, increases in diameter, may indent in one or two regions and finally, if the extract is sufficiently strong, vanishes completely. With strong extracts, or with concentrated sperm suspensions, disappearance of the membrane will occur in about three minutes if the gelatinous coat of the egg is present or in less than half a minute if the coat is first removed.

2. *Follicle cell dispersal by hyaluronidase.* The unfertilized tubal egg of mammals (with some exceptions such as the sheep, opossum and monotremes) is surrounded by a layer of follicle cells, the cumulus oöphorus, that persists for a considerable length of time. The cells are held together by a viscous material derived from the follicular fluid and the egg is thus covered by a tough coat through which the sperm must pass

to effect fertilization. It has been shown by Yamane (248, 249) and Pincus (171, 172) that when unfertilized tubal eggs of the rabbit are exposed to dense sperm suspensions or to saline extracts of the sperm, the follicle cells are dispersed within a few minutes. This effect could also be brought about by sperm of other mammals. At about the same time, and quite apart from the above-mentioned experiments, Duran-Reynals (32) and McClean (129) discovered what they called a spreading or diffusing factor in extracts of mammalian testes. This factor, when injected into the skin along with some indicator (dyes, India ink, hemoglobin, toxins, viruses) permitted widespread diffusion of the material which would otherwise be restricted to a small region. Used with a vaccinia virus a considerable enhancement of vaccinal infection occurred. Extensive investigations by these workers and others showed that the spreading factor was obtainable from various bacteria, snake venoms, poisonous insects, leech tissues and other sources besides mammalian testes and sperm (see reviews by Duran-Reynals, 33 and Meyer, 136), and have emphasized the importance of this factor in infection. Working along somewhat different lines, Meyer *et al.* (137) found an enzyme (called hyaluronidase) in autolysates of pneumococci, and later in extracts of rabbit iris, ciliary body and spleen, that hydrolyzed a polysaccharide (called hyaluronic acid) which he obtained from vitreous humor, umbilical cord and other sources. The latter two independent lines of work were correlated by Chain and Duthie's (13, 14) demonstration that testis extract also hydrolyzed hyaluronic acid, and subsequent work showed hyaluronidase activity in various other preparations containing spreading factor. The relation to fertilization came about through the discovery by McClean and Rowlands (131) and Fekete and Duran-Reynals (39) that the dispersal of the follicle cells of rat and mouse ova could be brought about by preparations of hyaluronidase from various sources besides mammalian testes, and that the active agent roughly paralleled the hyaluronidase in enzymatic and spreading action and in physicochemical properties.

The dispersal of the follicle cells results from a dissolution of the intercellular cementing material which is evidently hyaluronic acid, or at least similar to it. Considering simply the sperm extracts, the effect is relatively nonspecies-specific, cross-reactions being obtained with various species of mammals (e.g., bull and sheep sperm extracts on mouse ova). It is, however, rather tissue specific in that mammalian tissues other than the sperm yield little if any of the active agent.

3. *Dissolution of the gelatinous coat of sea-urchin eggs by hyaluronidase.* It was mentioned above in connection with antifertilizin (p. 194) that Hartmann, Schartau and Wallenfels (69) observed a disappearance of the gelatinous coat of the eggs of Arbacia under the influence of extracts of homologous sperm. In other species of sea urchins, Tyler and O'Melveny (226) accounted for a similar apparent disappearance by incorporation of the material of the coat in the precipitation membrane that is formed by interaction of the fertilizin of the coat with antifertilizin of the sperm extract and the rapid shrinkage of this membrane to the surface of the egg. In recent experiments, Monroy and Ruffo (140, 180) again report a dissolution of the coat of eggs of several species of sea urchins found at Naples (Psammechinus micr., Arbacia lixula and Spharechinus gran.) under the influence of a homologous or heterologous sperm extract prepared in the manner employed for extraction of hyaluronidase

from bull testis. They also find that bull testis extract causes a similar initial swelling of the coat but no final dissolution, and that the sea urchin sperm extract has viscosity-lowering action on hyaluronic acid preparations but is much less active in that regard than is the bull hyaluronidase. They conclude that sea-urchin sperm contain a hyaluronidase-like substance and the difference from bull testis-hyaluronidase is due to difference in relative amounts of two components of hyaluronidase. While there is evidence for the presence of two or more enzymes, or enzymatic activities, that may differ in relative concentration in preparations from different sources, the situation is not as yet well enough understood (see Meyer, 136) to permit ready application to the present case. Monroy and Ruffo also report no inhibiting action of egg water on the dissolution of the jelly coat by sperm extracts.

Runnström *et al.* (187) reported that hyaluronidase from bull testis did not attack the coat of eggs of the sea urchins P. miliaris and E. cordatum. They do not mention whether or not there was any swelling such as Monroy and Ruffo report. In this laboratory bull testis-hyaluronidase exhibited no dissolving or swelling action on the coat of eggs of L. pictus (221, 93) nor was such action obtained with the sea-urchin sperm extracts, prepared according to the methods used for hyaluronidase. When tested by the mucin-clot method these sea-urchin extracts showed no hyaluronidase activity. They also showed no spreading activity in guinea-pig skin and similarly negative results were obtained with a Megathura sperm extract containing the egg-membrane lysin. Another finding that is of interest in this connection is that concentrated preparations of sea-urchin fertilizin give a mucin-clot reaction (93). This reaction consists in the formation of a fibrous clot or a flocculent precipitate, depending upon the degree of polymerization or aggregation of the material, when protein is added to it in acid solution. Hyaluronic acid preparations give this reaction (138) but not after they are treated with hyaluronidase, which can then be assayed by this method (see Meyer, 136). That fertilizin, too, gives this precipitation reaction means only a very general resemblance to hyaluronic acid, since many mucins of acidic nature behave in this manner. As yet no inhibition of this reaction of fertilizin has been obtained with bull testis-hyaluronidase, and sea-urchin sperm extracts tend to enhance rather than to prevent it.

It is evident that further work will be required to ascertain definitely the presence or absence of hyaluronidase in sea urchins.

4. *Egg surface (vitelline-membrane) lysis.* Runnström *et al.* (183–189) have discovered an interesting new effect of methanol extracts on sea-urchin sperm. This is interpreted as a liquefaction of the egg surface and is manifested in the following ways: *a*) when placed in hypertonic solution the treated eggs shrink with a much smoother surface and cytolyze more readily than do the controls; *b*) cytolysis in hypotonic solution occurs in less dilute solution than is needed for the control eggs; *c*) the constriction into two fragments that occurs upon high speed centrifugation takes place more readily in the treated eggs; *d*) eggs of P. miliaris and E. cordatum, which are often quite elongate when shed, become spherical very soon after addition of the sperm extract. In the intact, unfertilized sea urchin egg the vitelline membrane is not readily discernible, but various observations of these authors strongly indicate that it is this structure that is attacked by their sperm extracts. Other effects of this

material include inhibition of fertilization in P. miliaris, inhibition of motility of sperm of E. cordatum, but not of P. miliaris, inhibition or retardation of agglutination of E. cordatum sperm by homologous egg water and by antiserum and sphering of mammalian erythrocytes. The authors find that most of these effects can be imitated by certain detergents (Duponol and Dupont QB), and by honey bee venom.

B. CHEMICAL AND ANTIGENIC PROPERTIES. 1. *Egg-membrane lysin.* Some of the properties of the lytic agent in extracts of Megathura sperm have been examined by Tyler (200). It is nondialyzable, salts out in saturated ammonium sulphate, is not extractable by alcohol and is readily inactivated by crystalline trypsin or chymotrypsin. It seems fairly safe to conclude that the lysin is of protein nature. It is extremely heat labile; at 50° C., for example, its half life is about one minute. Solutions prepared by ammonium sulphate precipitation and dialysis possess antifertilizin activity and the latter remains unaltered when the lysin is heat-inactivated. This does not necessarily mean that there are two distinct, separate-acting substances present. Considerably more information will be required before definite conclusions may be drawn in this regard.

Antiserum prepared in rabbits against active or inactivated preparations of the lysin precipitate with the solutions and render them inactive for membrane lysis (221). Whether or not they also neutralize the antifertilizin is not readily determinable, since the antiserum agglutinates the sperm. Cross reactions with abalone sperm were not obtained. The results show that the specific structures causing lysis are not really antigenic. This is consistent with the results of similar, more extensive investigations with hyaluronidase (see below).

2. *Hyaluronidase.* Work on the chemical properties of this agent has been reviewed by Meyer (136). In the present connection it should be emphasized that heat labile proteins are again involved. This is shown primarily by their inactivation by pepsin and trypsin, but not carboxypolypeptidase, nondialyzability and precipitation by many reagents commonly employed for proteins. At 60°C. solutions of bull testis-hyaluronidase at pH 7 lose 99 per cent of their activity in five minutes, 99.9 per cent in 15 minutes, and at room temperature activity is very rapidly lost below pH 4 and above pH 10 (127). Chemically purified preparations were found to be electrophoretically and ultracentrifugally inhomogeneous, the active component being isoelectric at about pH 5.7 and having a sedimentation constant (S_{20}) of 4.3 (62). Detectable hydrolytic or spreading reactions are obtained with as little as 10^{-4} mgm. of purified material.

There is evidence that hyaluronidase from different sources may differ enzymatically (see Meyer, 136). While all cause an initial depolymerization as shown by viscosity reduction (and presumably also by spreading and mucin clot tests), they differ in the extent to which they complete the hydrolysis of the hyaluronic acid into its acetyl glucosamine and glucuronic acid components. Testicular and leech hyaluronidase give only about half the theoretical yield (measured as reducing sugars), while pneumococcal hyaluronidase gives almost complete hydrolysis and can supplement the action of the testicular and leech materials. Apparently there are different enzymes (or different active groups on the same molecule) involved in the depolymerization and in the hydrolysis of the aldobionic acid. Hahn (63) has re-

ported separating two such enzymes from testicular extracts, as well as an acetylglucosaminidase which is not related to hyaluronidase activity.

Numerous investigators have shown that the activities of hyaluronidase can be blocked by means of specific antisera (see Duran-Reynals, 33; Meyer, 136). In contrast to the lack of species-specific action of hyaluronidase, the antisera are highly specific. Thus, antisera against the hyaluronidases of Clostridium welchi and Cl. septicum do not cross-react (130). In tests of dispersal of follicle cells of rat ova, antisera prepared in rats against bull testis-hyaluronidase did not cross-react with the rat testis enzyme (103). It may be concluded that the enzymatically active groups of the hyaluronidase are nonantigenic. Neutralization by homologous antibody may then be attributed to precipitation of the enzyme (which might also be linked to some antigenic protein) or blocking of the enzymatically active regions of the molecule by the presence of antibody combined with adjacent antigenic groups, or both.

3. *Egg-surface lysin.* Preparation of this substance according to the latest method described by Runnström and Lindvall (183) involves methanol extraction of frozen-dried sperm, solution in chloroform, absorption on zinc carbonate column which separates it from accompanying yellow coloring matter, elution in ethyl alcohol and repeated solution in distilled water and evaporation at 80°C. The substance is heat stable and dialyzes through cellophane.

The chemical properties of the egg surface lysin are quite different from those of antifertilizin. Its ability to neutralize fertilizin might imply some similarity in constitution or it might even represent the determinant groups of the antifertilizin molecules. Against this is the observation that it is not in turn neutralized by fertilizin and that it is relatively nonspecific. It seems more likely that it inhibits the agglutinating action of fertilizin by some nonspecific action such as tying up calcium, the absence of which interferes with the agglutination of sea urchin sperm. Its other effects seem to be more direct and lack of calcium, for example, has an effect opposite to that of the lysin in the hypertonicity test (186).

C. Role in fertilization. 1. *Egg-membrane lysin.* The manifest action of the lysin on the membrane barriers in molluskan and amphibian eggs implies that it is instrumental in enabling the individual spermatozoön to penetrate to the surface of the egg proper. For this purpose the membrane need not be completely dissolved. The membrane of eggs of this type remains visibly intact when penetrated by one or a few spermatozoa which evidently cause a small, hardly discernable lesion. Loeb (121) developed a theory of fertilization that postulated the presence of a lysin in sperm along with a corrective factor. The lysin was supposed to act on the surface of the egg and would lead to its destruction but, being checked by the corrective factor, results in activation. The membrane lysin cannot be considered a lytic agent of this sort. It has no effect on the surface of the egg proper, does not cause artificial activation nor affect the development of fertilized eggs (200). There have been reports (35, 164) of artificial activation of amphibian eggs by microinjection of sperm extracts but, since such eggs can be activated by simple puncture and by chemical means (see 205), it cannot be concluded that the extracts contained the particular activating agent of the sperm.

Since the egg membrane lysin is carried by the spermatozoön and probably enters

the egg with it, there is certainly the possibility that it participates in other activities, but there is as yet no evidence of further action beyond that described.

2. *Hyaluronidase.* The follicle cell mass of the mammalian egg is of tough consistency and evidently constitutes a barrier which would be difficult for the spermatozoön to traverse by mechanical means alone. The demonstration that the cementing material (probably hyaluronic acid) is dissolved by hyaluronidase, which the spermatozoön carries, provides a ready explanation of its ability to penetrate this barrier. Since the hyaluronidase that the sperm carries evidently functions in this manner it has seemed reasonable to deduce that addition of solutions of hyaluronidase to nonfertilizing quantities of sperm would effect fertilization. Experiments along this line have been reported by Rowlands (179) and by Kurzrok, Leonard and Conrad (100). According to the latter workers, fertilization was obtained in six human females, with a history of sterile marriage, when bull testis-hyaluronidase was added to the semen specimen used for artificial insemination or placed in the genital tract prior to coitus. Since appropriate controls are not readily feasible in experiments of this type with humans, the effect of hyaluronidase on the results is difficult to assess. Rowlands reports a series of seven experiments with rabbits. In two experiments the number of spermatozoa required for fertilization was checked and found to range from 4.5×10^5 to 1.8×10^6. In the other five experiments hyaluronidase (filtrate from 2×10^7 sperm) was added to the inseminates and increased fertility was obtained in four of them. The median effective concentration of sperm in these range from 5.4×10^4 to 1.5×10^5, and the average increase in fertility is such that one-sixth the normal amount of sperm is needed when the enzyme is present, the range being from one-thirtieth to equal concentration in different tests.

There is, then, evidence that hyaluronidase enhances fertilization in mammals. Since it is added in solution it would appear that it acts by contributing to the dispersal of the follicle cells. This would support the previous explanation, offered by McClean and Rowlands (131), of the large number of sperm required for fertilization in mammals. The additional sperm are presumably required to break down the follicle coat of the egg. This implies that a single spermatozoön does not carry enough enzyme for this purpose, not even enough to make a little pathway for itself. Since enzymes are not ordinarily used up in the reactions that they catalyze, it might be supposed that in time a single spermatozoön would manage to break down the coat sufficiently to get through. But this might involve a longer time than its life span would allow. There are, of course, other possible explanations for the apparent failure of a single spermatozoön to get through the coat unaided. Thus there may be loss of hyaluronidase from the spermatozoa in transit and inactivation of dissolved hyaluronidase with time at body temperature. It has been shown that the hyaluronidase content of semen is proportional, within limits, to the sperm concentration (240, 100). The hyaluronidase is evidently given off continuously by the spermatozoa and this is apparently aided by secretions of the seminal vesicles, since the latter tissue, when incubated with testis homogenate, gives an increased yield of hyaluronidase (104). In the genital tract of female rabbits the ratio of hyaluronidase to spermatozoa was found to decrease with time (61) and this has been interpreted to mean that 'secretion' of the enzyme ceases there. However, to establish that

point it would be desirable to know how the activity of hyaluronidase solutions, introduced into the genital tract, varies with time. In artificial insemination experiments the added hyaluronidase must evidently reach the eggs and it is, then, of importance to learn how its transportation up the genital tract is accomplished. It is also of interest to know to what extent the follicle cell mass must be dispersed in order to effect successful fertilization. By way of comparison, a striking example of fertilization without loss of follicle cells may be mentioned. The eggs of ascidians are surrounded by a layer of so-called test cells, covered by a continuous membrane on which is another layer of follicle cells. The spermatozoön must penetrate through these cells, or the intercellular cement, and through the membrane (which has no pores large enough for the sperm). This is accomplished without apparent effect on these coats which persist until the developing embryo is ready for hatching.

It should be emphasized that it is not only in mammals but in practically all animals that large numbers of spermatozoa must be present in order to effect fertilization. One reason for this is quite evidently the necessity of providing sufficient opportunity for sperm and egg to encounter within their respective life span. Another factor is the proportionate incapacitation of sperm by their reaction with specific egg substance in solution in the medium (see p. ooo). Another factor may be the occurrence of large numbers of physiologically defective sperm, but there is as yet no specific evidence concerning this. In any event, such factors may also be expected to be involved in fertilization in mammals. The great interest that has been aroused by hyaluronidase will undoubtedly result in experiments designed to answer these and other questions relating to the precise manner in which it is involved in the fertilization process.[2]

3. *Egg-surface lysin.* The lytic agent postulated by Loeb (121) might appear to be represented by the surface-liquefying agent that Runnström *et al.* (183–189) extracted from sperm. Runnström has mentioned (personal communication) that the substance is an unsaturated fatty acid. It would thus resemble agents known to initiate activation and also cause lysis in sea urchin eggs. Runnström *et al.* (188) consider their results to offer some support for Loeb's lysin theory of activation.

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[2] While the present article was in press papers dealing further with these questions have been published by G. I. M. Swyer (Biochem. J. **41:** 409, 1947 and **41:** 413, 1947), by M. C. Chang (Proc. Soc. Exp. Biol. Med. **66:** 51, 1947) and by S. L. Leonard, P. L. Perlman and R. Kurzook (Proc. Soc. Exp. Biol. Med. **66:** 517, 1947). Swyer finds the hyaluronidase content of semen of various mammals to be directly correlated with sperm count. He also demonstrates that hyaluronidase is not actively manufactured by the ripe spermatozoa, but simply dissolves off at various rates depending upon conditions. The rate of liberation of the enzyme is found to increase with dilution of the suspension and to decrease upon addition of hyaluronidase to the suspension.

The other two papers further justify the doubts expressed above as to the necessity for dispersal of the follicle cells. Chang finds no enhancement of fertilization due to addition of hyaluronidase in rabbits. He interprets Rowland's positive results as due to the action of the seminal fluid present in his hyaluronidase preparations since that fluid, free of hyaluronidase, is found to be capable of maintaining the fertilizing capacity of the sperm. The observations of Leonard *et al.* on rats show that fertilization normally takes place before there is any distinct dispersal of the follicle cells and that introduction of hyaluronidase into the uterine horns of rats in heat results in no denudation of tubal eggs. These papers support, then, the view that the individual spermatozoon carries enough hyaluronidase to effect its penetration through the follicle coat of the egg.

However, there is no evidence that their lytic agent induces membrane elevation or causes general cytolysis upon prolonged treatment of the eggs except perhaps in very high concentration. Hypertonic sea water, which is a corrective agent according to Loeb, has the reverse action in connection with Runnström's lytic agent. Runnström *et al.* (188, 183) have also expressed the view that the lytic agent may be responsible for the establishment of the block to polyspermy. They note that it is obtainable from eggs as well as sperm and so could be released in the egg surface upon attachment of a single spermatozoön. Since the agent inhibits agglutination they consider it to be effective in inhibiting the attachment (agglutination to the egg surface) of supernumerary spermatozoa. It should be remarked, however, that supernumerary sperm attach themselves to the surface of eggs and remain (attached?) there after fertilization. That part of the hypothesis appears then unnecessary and they could appeal simply to the demonstrated fertilization-inhibiting action of their lytic agent to support the view that it is instrumental in preventing polyspermy, provided that there is evidence of sufficiently rapid release and action on the egg surface.

That materials with detergent-like action can be extracted from sea urchin sperm and eggs is of decided interest, but before speculating on their possible role in fertilization, it is desirable to know whether they are available under physiological conditions. Runnström *et al.* (188) report preliminary tests showing that the agent is present in the supernatant obtained by centrifugation of a heavy suspension of live sperm or frozen and thawed sperm. They conclude that it is rather loosely bound to the spermatozoa. Presumably, too, it is not given off by living spermatozoa in sufficient amount to render the egg nonfertilizable before the spermatozoa reach it.

4. *Self-sterility in Ciona.* In connection with egg-membrane lysins, as well as the other specific substances of eggs and sperm, the phenomenon of self-sterility in the hermaphroditic ascidians should be discussed. In recent years this has been the subject of extensive investigations by T. H. Morgan (145–156), who is also responsible for most of the early work on this topic, and by H. Plough (173–175). Only a brief review of such features as pertain to sperm and egg extracts will be attempted here. In many hermaphroditic animals including the ascidians the eggs are more or less refractory to sperm of the same animal. This is strikingly exhibited in the ascidian Ciona in which a sperm suspension that gives 100 per cent cross-fertilization (with eggs of another individual) will, even when over 200 times more concentrated, generally yield little if any self-fertilization. The absolute amounts of sperm required varies in different individuals and, in all cases, increasing the concentration of sperm increases the percentage of self-fertilization, but only rarely can 100 per cent selfing be obtained. It is evident that self-sterility is a relative affair. For brevity, however, we shall use the term without qualifying adjectives. Cross-insemination tests with wild individuals show occasional cases of cross-sterility and these occur more frequently among offspring of a self-fertilized individual or of a single pair of individuals. Morgan showed that the situation has a genetic basis, although the exact factor-analysis has not as yet been accomplished. He also demonstrated by various ingenious experiments that the block to self-fertilization resides in the membrane (between test and follicle cells) that surrounds the egg. This membrane

and the adhering cells can be removed mechanically (with some difficulty) or by means of proteolytic enzymes, and the eggs are then self-fertilizable. It is not, however, necessary to remove the membrane. Treatment of the eggs for five minutes with pH 2.7 sea water has no visible effect on it, but renders the eggs self-fertilizing.

The block to self-fertilization is evidently of highly specific nature since the gametes of almost any pair of individuals selected at random fertilize readily. The materials on which it depends must, then, be of a highly specific nature. In some early experiments by Fuchs (45) egg water and egg extracts were found to enhance the cross-fertilizing power of Ciona sperm. Morgan extended the experiments to self-fertilization with tests of various kinds of egg and sperm extracts. For the most part no marked effects were obtained. The presence of, or treatment with, self-egg water was found (145, 146) to reduce the self-fertilizing, but not the cross-fertilizing capacity of the sperm. However, in the cross-fertilization tests an excess of sperm was generally present and occasional experiments did show inhibition. Foreign egg water gave no increase in self-fertilization. Sperm extracts would cause a slow (24 to 48 hours) breakdown of the membrane in many cases and this seemed to occur more readily with foreign eggs than with eggs of the same individual. No significant facilitation of selfing nor inhibition of crossing was obtained by use of the sperm extracts.

There is, then, some indication that lytic and specific interacting substances may be extracted from Ciona sperm and eggs. The relative weakness of the extracts may be attributed to various factors, such as methods of extraction, instability, etc. The facts of self-sterility preclude the possibility that the lytic agent is simply a proteolytic enzyme, although it is possible that some such enzyme may be released by the interaction of the specific substances of the egg and sperm. These substances must evidently possess a degree of specificity of interaction that is as great or greater than that exhibited in the human blood groups.

GENERAL CONCLUSIONS

From the work reviewed here it is evident that fertilization involves the interaction of a number of substances on and within the eggs and sperm. Certain of these substances interact in the manner of antigen and antibody. Others have enzymatic activities and others even may have detergent-like action. The general chemical nature of certain of these agents has been established. Their precise role in fertilization has not as yet been determined, but the evidence shows them to be concerned in the initial steps, including the penetration of coats and membranes surrounding the egg and attachment to the surface. The action of these and other substances in the further steps, involving incorporation of the spermatozoön into the egg and activation of the latter, is for the most part unknown. The specificity of interaction of these substances provide a basis for understanding the tissue and species-specificity of fertilization, although none of the specificities so far examined show precise correspondence. Various properties of the interacting substances provide analogies with phenomena in the fields of immunology and virology. These include such features as blocking antibodies, heteroagglutination, zone phenomena, phage neutralization, bacterial invasiveness, etc. Concepts employed in these fields find application in

studies of fertilization and these in turn suggest new general views, such as the auto-antibody concept of cell structure that is briefly discussed.

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CHEMORECEPTION IN INSECTS

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HIGHLY SPECIALIZED SENSORY RECEPTORS normally capable of being excited by direct contact with chemicals attain their most spectacular development in two groups of animals, the insects and the vertebrates (cf. von Frisch, 59). Despite radical histological and chemical differences in the superficial structure of the end organs within the two groups, there appear to be striking physiological similarities in the behavior of each toward numerous chemical compounds and in the manner in which stimulation is presumed to be mediated. This apparent paradox has not only stimulated interest in the study of insect chemoreceptors but has also promoted a wealth of confusion by reason of anthropomorphic interpretation. Nevertheless, it is becoming increasingly apparent that chemoreception throughout the animal kingdom is basically similar and that much is to be gained by the study of forms, such as insects, which frequently lend themselves more advantageously to experimentation than does man. The present review, therefore, considers chemoreception in insects, not as an isolated physiological phenomenon, but as one facet of the intricate relation between animal chemoreceptors and the compounds which excite them.

DISTINCTIONS BETWEEN TASTE AND SMELL

It is now generally accepted that the chemical senses of insects may be separated into three categories which correspond roughly to smell, taste and the common chemical sense of vertebrates (39, 59, 103, 104, 145). However, the criteria upon which this division is based are at least as tenuous and unsatisfactory as those applied to vertebrates (73, 96, 111). The following well-known points advanced to permit of distinction between the gustatory and olfactory senses of vertebrates are here re-examined in the light of newly acquired data on the physiology of invertebrate chemoreceptors (59, 73, 103):

1) the two types of end organs differ anatomically and with respect to their distribution in the body; 2) olfaction is subserved by primary neurones and gustation by secondary neurones; 3) the two senses differ with respect to their threshold concentrations; and 4) compounds which stimulate one set of receptors usually do not stimulate the other set.

1) The adequacy of anatomical and topographical factors as valid criteria in insects suffers on two counts. Although the distribution of chemoreceptors has been the subject of heated controversy since 1798, the great body of accumulated experimental evidence is inadmissible by reason of questionable techniques (cf. Mar-

shall, 96). For this reason and because new loci of chemical stimulation are still being discovered (40), it is not yet advisable to relegate each chemical sense to specific areas of the body. A second difficulty is imposed by the fact that the end organs subserving both senses frequently occupy the same segments of the same appendage (e.g., the antennae of honeybees and the labellum of muscoid flies). It follows, therefore, that nerve fibers from both types of receptors are contained in the same nerve wherefore they enter the same lobe of the brain. In the case of the antennal nerve of honeybees this would be the deutocerebrum. Here the fibers even follow the same tracts inasmuch as all terminate in glomeruli situated principally in the deutocerebral neuropile. Sensory fibers from the labellum are gathered into the labial nerve, which enters the suboesophageal ganglion. Hence no analogy can be drawn with vertebrates where olfactory receptors are innervated by the olfactory nerve and taste receptors by the chorda tympani, glossopharyngeal and vagus (7, 81, 115, 120, 154).

2) As Hanström has pointed out, the histological criteria applied to vertebrates are inadequate here because all receptors in insects are primary sense cells (cf. Snodgrass, 128). Moreover, histological information sufficient to permit a comparison on the basis of any other points of finer structure is still incomplete, since considerable doubt surrounds the identity of the chemoreceptors in many insects.

3) Quantitative differences based upon threshold values constitute the most promising point of comparison. Parker and Stabler (112) have shown that the minimum concentrations of ethanol perceptible by each of the three chemical senses in man are: for smell 1.25×10^{-4} M, for taste 3 M, for the common chemical sense 5 to 10 M. Comparable values measured as rejection thresholds have been determined for insects. Ethanol acting as a gas on the antennal and labial end organs of female houseflies is rejected at 5×10^{-3} M (144). Acting as an aqueous solution in 0.1 M sucrose on the tarsal receptors of blowflies it is rejected at a concentration of 3.2 M (42). Both values should, of course, be obtained for the same species; however, the similarities between the sensitivities of the tarsal receptors of unrelated species indicate a strong likelihood that this comparison between two closely related flies may be a valid one.

The relative sensitivities of taste and smell are more strikingly illustrated when the limits in each category are examined. For man, one of the most effective taste substances is quinine hydrochloride. It is perceived in concentrations which are approximately 1.5×10^{-7} M (108). Aquatic beetles may be conditioned to it at dilutions of 1.25×10^{-6} (9). One of the most effective olfactory stimuli for man, a mercaptan (probably methyl), is perceived in concentrations which are approximately 9×10^{-13} M. Values for this or similar compounds have not, however, been obtained with insects. Values which approximate the limits of sensitivity for the two categories of receptors have been obtained with a few other compounds. The tarsal receptors of some butterflies (e.g., *Danaus menippe*) can be stimulated by solutions of sucrose as dilute as 9.8×10^{-6} M (5); those of some flies (*Calliphora vomitoria* and *Cynomyia*) by 3.9×10^{-5} M sucrose (27, 105). Carefully conducted experiments designed to test the sensitivity of olfactory receptors have been few. Warnke (140) has found that dung beetles of the genus *Geotrupes* respond to the odor of a 2.3

$\times 10^{-8}$ M skatol gas. The tent caterpillar, *Malacosoma americana*, is repelled by a gaseous mixture of benzaldehyde equivalent to 4.1×10^{-7} M (39). In neither case, however, do these values represent the limit of sensitivity for it is known that insects can be stimulated by incredibly low concentrations of substances in the gas phase. By way of illustration, odors, probably lipoid, protein or ester (23, 72) emanating from female gypsy moths attract males from distances as great as $2\frac{5}{16}$ miles.

That a strict distinction between taste and smell cannot be drawn even on a quantitative basis is illustrated by a comparison of thresholds of the most stimulating taste and smell substances for man (63). When these are compared in terms of molecules per cubic millimeter, a continuous spectrum and overlapping of stimulating effectiveness is observed from the most effective olfactory substance (trinitrobutyltoluene at $1.03–2.06 \times 10^4$ molecules per cu. mm.) through one of the least effective (nitrobenzene, 2.006×10^{11}) to the most effective taste substance (quinine sulfate, 2.42×10^{11}). Nevertheless, the fact is inescapable that taste is on the whole less sensitive than olfaction.

4) The proposition that that which is smelled is usually not tasted and vice versa (111) is even less applicable to insects than it is to vertebrates. While it is true that sugars and many inorganic compounds (e.g., salts) which stimulate insect gustatory receptors apparently fail to stimulate olfactory receptors, many organic compounds (viz. alcohols, aldehydes, fatty acids, etc.) as solutions, stimulate oral and tarsal receptors (22, 42) and as gases stimulate olfactory receptors (24, 144). Two crucial experiments which would test this proposition have not been undertaken with insects and with vertebrates have yielded controversial results. It is not known for insects whether solutions which stimulate gustatory receptors can, as solutions, stimulate olfactory receptors nor whether compounds which in the gas phase stimulate olfactory receptors can do so equally well in the liquid phase.

Kunze (90) and Marshall (97) have shown that the antennae of the honeybee can be stimulated both by 'mild odors' and by solutions of sucrose or certain other taste substances. The antennae bear, however, innumerable sensilla of several different types, and there is no proof that the same receptors are responding in each case. Thus this cannot be construed as evidence to support a hypothesis that there is no rigid barrier between the concepts of gustation and olfaction.

Despite the inadequacy of the foregoing criteria it must be concluded that there is as much ground for separating taste and smell in insects as in vertebrates. In terrestrial insects and those aquatic species which are adventitiously terrestrial there are clearly two categories of receptors involved. The olfactory receptors react to very low concentrations of compounds which are volatile at ordinary temperatures; the gustatory receptors react to higher concentrations of liquids or solutions which may or may not be volatile at room temperature.

With regard to aquatic species the situation stands in need of clarification by additional experimental inquiry. While it appears that two categories of receptors are involved here also, the experiments of different workers are contradictory in several important respects. In each study, water beetles were conditioned to one of several compounds arbitrarily designated as odor substances (artificial musk, coumarin,

heliotropin and skatol) or to one of several taste-substances (sucrose, NaCl, HCl and quinine hydrochloride). According to Schaller (125) *Dytiscus* perceives odors under water by means of the nine funicular segments of the antennae and by the tips of the maxillary palpi. Out of water the same compounds stimulate as vapors. Taste substances, on the other hand, are perceived by the submerged beetle through the maxillary palpi, the labial palpi and the inside of the mouth. According to Ritter (122) *Hydrous piceus* bears olfactory receptors on the apices of the maxillary palpi only. Sucrose, NaCl and quinine salts stimulate organs on the tips of the maxillary palpi while stimulation by HCl is restricted to the labial palpi. Bauer's (9) results are in disagreement in that he failed to prevent responses to sugars, salts and bitter substances by removing the maxillary palpi of *H. piceus*.

Localization of the sour taste on a different appendage from that bearing the other taste organs, if indeed true, casts further doubt on the wisdom of attempting to distinguish taste from smell solely on anatomical grounds. Moreover, the conditions of all of the experiments cited above precluded the accurate control of concentrations; hence, the criterion of sensitivity cannot yet be applied to the separation of taste and smell in aquatic species. Furthermore, at least two of the 'odors' tested, heliotropin and coumarin, stimulate the gustatory as well as the olfactory sense of man and in the case of the aquatic beetles may conceivably have acted upon receptors other than olfactory.

Experiments with fishes have indicated that water solutions of compounds which are taste substances for terrestrial vertebrates act as taste substances for aquatic vertebrates also, while compounds which in aqueous solution are olfactory for fishes are olfactory for terrestrial animals (58, 59). If the results of Schaller, Ritter and Bauer can be reconciled one with the other, they constitute grounds for extending this parallel to insects.

With wireworm larvae (*Agriotes* spp.), which burrow in moist soil, the response to chemical stimulation is essentially like that of aquatic organisms. There are clearly two categories of stimulating compounds. Low concentrations of aqueous solutions of several dibasic acids, amino acids and amides elicit an orientation response. Solutions of sugars, triolein and animal proteins in high concentration elicit both an orientation response and a biting response. Neither group stimulates in the gas phase (136). The concentrations necessary to evoke a biting response are said to be comparable to those usually adequate for organs of taste while effective concentrations in the case of those compounds which elicit only an orientation response are of the order characteristic of olfactory stimulation. For example, the activity (negative logarithm of concentration) of aspartic acid is 11; that of triolein, 2. Recent experiments (26) place the receptors on the antennae and galeae of the maxillae. The former are large sensilla basiconica; the latter, small sensilla basiconica. Stimulation of the maxillary organs by compounds of the second above-mentioned category (e.g., two per cent glucose) provokes both orientation and biting responses while stimulation by compounds of the first category (e.g., asparagine at 10^{-3}–10^{-9} mgm./l.) provokes only an orientation response. On the other hand, stimulation of the antennal organs either by two per cent glucose or 10^{-3}–10^{-9} mgm./l. asparagine provokes only orientation. It is difficult to interpret these results in terms of taste and

smell, and the authors of the experiments scrupulously avoid doing so. At best it can be pointed out that both categories of compounds stimulate both types of receptors, and that the animal may be able to distinguish between compounds of the two categories when they stimulate the maxillae but not when they stimulate the antennae.

Solely for convenience of discussion, therefore, we shall consider the gustatory sense of terrestrial insects as that which is mediated by chemical stimuli acting as liquids or solutions at relatively high concentrations on contact and the olfactory sense as that mediated by chemical stimuli in a gaseous state at relatively low concentrations.

COMMON CHEMICAL SENSE

It can no longer be doubted that insects possess a third category of chemoreceptors in addition to and distinct from olfactory and gustatory receptors. Adequate stimuli are high concentrations of numerous so-called irritating compounds of which ammonia, chlorine and essential oils are examples (74). Early failure to recognize the existence of a common chemical sense was largely responsible for the contradictory results which for a century and a half stemmed from experiments designed to locate the olfactory organs (cf., e.g., 93, 94).

It appears that several classes of compounds can stimulate both the olfactory and common chemical senses. Insects which have been conditioned to weak odors of essential oils lose their ability to recognize these odors after the olfactory receptors are extirpated (51) but continue to exhibit violent avoiding reactions if the concentration is increased to maximal. Mealworm beetles fail to respond to odors from food or the opposite sex if the antennae are amputated, but responses to the vapors of essential oils persist. Unilateral amputation results in circus movements toward the intact side if normal odors are presented. When the vapors of essential oils are used as stimuli, circus movements are away from the intact side (137). In this connection the observations of Weyrauch (143) concerning the earwig *Forficula auricularia* L. are interesting. Food odors directed to the antennae of this insect elicited no response, but strong artificial odors did. On the other hand, food odors evoked responses when directed to the maxillary palpi. Following amputation of the palpi these responses ceased. Weyrauch concluded that the maxillary palpi mediated positive chemotaxis; the antennae, negative chemotaxis.

Few quantitative experiments dealing with the common chemical sense have been recorded. Dethier (38) demonstrated with *Pieris* larvae that the threshold of response to the odor of benzaldehyde is increased 17 times following removal of the olfactory receptors; however, no attempt was made to localize the sensitive region.

This much can be said with certainty regarding the common chemical sense. For excitation, a high concentration of stimulus is required, and the response is always in the nature of an avoiding reaction. The receptors have not yet been located.

OLFACTORY SENSE

Adequate stimuli for olfaction in terrestrial insects are the vapors of compounds which volatilize at physiological temperatures. Literally thousands have been in-

vestigated (cf. Dethier, 39), albeit under pressure of economic needs. The behavioristic aspects of olfaction have been adequately treated by Fraenkel and Gunn (50) and Dethier (39), but the physiology of olfaction has been less extensively studied.

1. *Receptors*

Olfactory receptors in insects are located chiefly on the antennae (96, 145). They are, in addition, commonly found on the maxillary and labial palpi or their homologues. These sites of action have been discovered through observations of the ability of insects from which one or more appendages have been extirpated to orient to food or sex odors or odors to which they have been conditioned. In evaluating localization experiments of this sort it has been necessary to take into consideration possible deleterious effects attendant upon destruction of stimulatory organs. Some insects bear in the antennae such stimulatory organs (153) whose somewhat secondary proprioreceptive function is to assist in the maintenance of muscular tone and in the initiation of muscle kinetics. Following antennal amputation these insects fall into a state of akinesis from which they are aroused only with difficulty (149). For many species there are no untoward effects following destruction of the various appendages.

In a few species the identity of the actual receptive surfaces involved has been determined experimentally. Previously function was ascribed on morphological grounds entirely. Three basic types of receptors have now been identified: pore plates (sensilla placodea) (57), thin-walled cones and pegs (sensilla basiconica, 11, 19, 38, 41, 140, 141, 146), and thin-walled pegs or cones sunken in pits (sensilla coeloconica, 11).

In his classic experiments von Frisch (57) trained honeybees to associate food with the odor of an essential oil. Bilateral amputation of the flagellar segments of the antennae did not impair this response as long as the proximal three-fourths of the fourth flagellar segment of one antenna remained intact. A histological survey of the antennal sensilla revealed that the pore plates alone coincided in distribution with the physiological results. Warnke (140) was able to demonstrate with dung beetles (*Geotrupes sylvaticus* and *G. vernalis*) that the areas of the antennae and palpi which were necessary for olfaction bore a profusion of sensilla basiconica peculiar to those areas. Dethier (41) showed that species of *Necrophorus* and *Silpha* were able to orient from distances to decomposing flesh as long as a small fraction of one antennal lamella remained intact. Again this bore a type of sensillum basiconicum not found elsewhere. With lepidopterous larvae he demonstrated that the antennae bore olfactory receptors and that of the four types of sensilla present the sensilla campaniformia were proprioceptive, the sensilla trichodea tactile and the sensilla basiconica olfactory. Wigglesworth (146) proved that the louse *Pediculus humanus corporis* de Geer loses its ability to respond to odor when the sensilla basiconica of the antennae are covered with cellulose paint, but that removal of the paint restores olfactory perception. Begg and Hogben (11) compared the olfactory responses of wild type *Drosophila melanogaster* with those of the mutants *antennaless* (A_o) and *aristapedia* (ss_a). *Antennaless* fails to respond to odors which normally lead the

flies to food, and *aristapedia* responds less strongly than do normal flies. A comparison of the antennae of the two forms (wild type and ss_a) indicated that either the sensilla basiconica or sensilla coeloconica or both are olfactory receptors. Bolwig (19) has proved that amputation of the dorsal papillae of the cephalic lobes of housefly larvae completely destroys the olfactory sense. These papillae are forms of sensilla basiconica.

All of these olfactory receptors possess in common a cuticular covering which is completely or partially thin-walled, innervation by a *group* of bipolar sense cells, and minute refringent bodies (Riechstäbchen) situated on the distal processes of the neurones (128). Nothing is known concerning the chemical or physical properties of the cuticle surmounting these receptors.

2. *Acuity*

Attempts have been made to assay the acuity of the olfactory sense and its powers of discrimination by determining threshold values. Such values are empirical inasmuch as it has been impossible thus far to determine the absolute threshold. This may be defined as the lowest concentration necessary to excite the most sensitive receptor element. As molecular concentration exceeds this amount a point is attained which may be termed the threshold of response. There is little doubt that this includes an expression of central nervous system phenomena. Whether it expresses itself initially as acceptance or rejection is predetermined by the genetic constitution of the animal as well as by its physiological state. It may be altered experimentally by such factors as preimaginal conditioning (134). If the threshold of response is manifested as a true rejection, the compound is rejected throughout the entire supraliminal range. Since the response frequently becomes more violent as the concentration in increased, it suggests that a different group of receptors, those of the common chemical sense, for example, may become involved. If a compound is initially accepted, it may become more attractive as the concentration is increased until an optimum is attained. Beyond this point attractiveness decreases with increased concentration till the nature of the response is completely reversed. Again it is probable that either a different set of receptors becomes involved or there is an increase in the frequency of impulses discharged by the receptors initially stimulated. For every attractant thus far tested there has been found a concentration in excess of which the substance is rejected. This has been illustrated by Dethier's analysis of data from Reed (121) for *Drosophila*, Folsom (48) for the Mexican boll weevil and Wieting and Hoskins (144) for the housefly.

Thresholds of response have been determined for a few odorous compounds with a small group of insect species. These values are given in the accompanying table 1. It is obvious that the olfactory acuity of insects and vertebrates cannot be compared on the basis of available data. On the strength of an insect's ability to orient to food, host, mate or nest by means of odors imperceptible to man, it is generally conceded that the insect sense has a vastly lower threshold. On the other hand, von Frisch (56) has demonstrated that the minimum perceptible odor of many essential oils is of the same order of magnitude for the honeybee and man. His experiments were based upon observations of the responses of conditioned bees to the odor of

various dilutions (in paraffin oil) of a test compound (essential oils). They were thus semiquantitative at most inasmuch as the concentration in terms of weight per unit volume of gas was unknown. Conditioning as a technique appears to be legitimate in that thresholds so determined depend in no wise upon the concentration to which the insect is conditioned (56).

All of the olfactory organs of insects are not equally acute. In general the thresholds for antennal organs are probably lower because many species can respond to distant sources of odor only so long as the antennae are intact. At the same time the ability to respond to stronger odors or those near at hand is not impaired as long as the palpi are intact (41, 51, 64, 140). Measurements of the threshold of response of larvae of the cabbage butterfly (*Pieris rapae*) for benzaldehyde before and after amputation of either the antennae or maxillae also suggest dual sensitivity (38).

TABLE 1. COMPARISON OF OLFACTORY THRESHOLDS OF CERTAIN INSECTS AND MAN

COMPOUND	MAN (RECOGNITION THRESHOLD, MGM./L.)	SPECIES OF INSECT (THRESHOLD OF RESPONSE, MGM./L.)		AUTHORITY
skatol	0.000,000,4	*Geotrupes sylvaticus*	0.003–0.009	Warnke (140)
		G. vernalis		
indol		" "	0.003	Warnke (140)
benzene	0.0053	*Habrobracon juglandis*	0.5–3.0	Wirth (151)
cyclohexane	0.25	" "	3–5	" (151)
ethanol	5.76	" "	5–20	" (151)
"		*Musca domestica*	230	Wieting and Hoskins (144)
ammonia	0.035	" "	0.04	" "
"		" "	1 (rejection)	" "
benzaldehyde		*Pieris rapae*	0.058	Dethier (38)

Before antennectomy or maxillectomy the threshold is 0.058 mgm./l. No change follows maxillectomy; following antennectomy the threshold is 0.077 mgm./l. Following combined antennectomy and maxillectomy the threshold rises to one mgm./l. This may represent a threshold of the common chemical sense.

3. *Factors Influencing Threshold of Response*

Any values obtained by an experimental method which utilizes some component of behavior to signal stimulation are subject to at least the same factors of variation as is the behavior itself. Such variations in behavior are discussed at some length by Fraenkel and Gunn (50). It is to be expected, then, that olfactory thresholds of response may be profoundly influenced by various environmental and internal physiological factors. Among the former are temperature and humidity.

Nearly all investigators working with olfactometers have found temperature and humidity control necessary. In most of the apparatuses response depended upon locomotion and orientation. Alterations of response were then traceable to the effects of temperature and humidity on the overall activity of the animal. This condition may also apply to feeding responses for which optimum temperatures and

humidities have been observed (2). Because of the relation between food and odors it is to be expected that responses to the latter may be altered in the same manner by the same variables. No evidence has been forthcoming to indicate that absolute thresholds in insects are affected by these factors, although for man it has been reported (152) that odor perception is more acute at low humidities than at high.

Internal physiological states which may affect thresholds of response are age, sex, state of nutrition and conditioning.

Records of age effects are few. Schwarz (127) has suggested, but not proved, that olfactory acuity decreases with the onset of senility. He offers this as an explanation of the fact that old individuals of certain moths frequently oviposit on plants unacceptable to the larvae. Bolwig (19) cites experiments to indicate that fly larvae in the first instar are less responsive to olfactory stimulation by normal food odors than are older larvae. He warns that the lack of response may be an artifact arising from the technique employed. He did prove, however, that housefly larvae suddenly change their behavior toward ammonia and other normally attractive substances three and one-half days after the second molt. At this time the larvae cease feeding, and the lateral portions of the ring gland undergo accelerated growth. If the ring gland is extirpated, feeding does not cease and pupation never occurs. Experiments in which the ring gland was removed and which were designed to indicate whether or not there existed a relation between the hormone and olfactory response were, unfortunately, inconclusive.

Effects which are associated in time with gonad development are observed also in the behavior of the parasite (*Pimpla ruficollis*) of the pine shoot moth (*Rhyacionia buoliana Schiff.*) toward essential oils found in its normal habitat (135). Emerging from its host long before the next generation of host larvae is available for oviposition, the parasite forsakes pine trees for Umbelliferae upon which it then commences to feed. At this time its ovaries are small, and oil of *Pinus sylvestris* is repellent. Later in the third or fourth week of life the ovaries enlarge, oil of pine becomes attractive and the females return to pine where hosts are now available.

The influence of sex upon thresholds of response is most evident in species where feeding habits are markedly different, e.g., species of mosquitoes of which the males feed on plant juices and the females on blood, but exact measurements of differences have been made only with *Drosophila* and houseflies. For the former, Reed (121) found that females exhibited a maximum response to ethanol and acetic acid at concentrations of 10 to 15 per cent and 0.4 per cent, respectively, while the maximum responses of males occurred at five per cent and 0.2 per cent, respectively. It was demonstrated in addition that these values represented differences in response to odor and not merely differences in activity. Wieting and Hoskins (144) found that female houseflies were more strongly attracted than males to ammonia, while the reverse was true with respect to ethanol.

Marked effects of nutritional states upon olfactory thresholds have not yet been demonstrated.

Conditioning, natural or experimental, may alter the threshold even to the point of reversing the sign of the response. Thus in some species, substances which normally elicit a rejection may be made to provoke acceptance. By preimaginal con-

ditioning Thorpe (134) has produced *Drosophila* adults which respond positively to the odor of peppermint. Crombie (25) has changed the response of blowflies to menthol from negative to positive. In every case, however, the change is of a transient nature lasting for a few days only. Moreover, there are limits to the changes which can be evoked. Though an insect may be conditioned to accept a normally repellent material, this conditioning does not survive if the concentration is too greatly increased. In the case of certain compounds conditioning is impossible. Von Frisch (56) could not condition bees to lysol, skatol and other compounds of of this type.

Although there are no very conclusive experiments proving adaptation to odors, a few suggestive observations have been recorded. Strebel (131) stated that Collembola exposed to stimulating odors became much less excited, as indicated by movements of the antennae and by general behavior, after being exposed to the same odor for some period of time. Wigglesworth (146) reported that the human louse shows adaptation in a constant field of stimulation. This conclusion is based upon observations of orientation in an odor gradient. If an animal should continue to proceed in a straight line in a favorable zone and followed a sharply convoluted course upon entering an adverse zone, it would become trapped in the unfavorable region. Actually, as a result of gradual adaptation, it makes increasingly long excursions before turning and finally goes straight. On reaching a favorable zone it continues to go straight. On the other hand, Wieting and Hoskins reported that the response of houseflies to an attractive concentration of NH_3 did not vary over a 20-minute period of constant exposure.

Finally thresholds of response may be altered if the animal is placed in a compound field of excitation involving antagonistic stimuli (25, 66). *Lucilia* exposed to light and the repellent odor of menthol illustrate this point. Crombie (25) placed flies in an olfactometer so constructed as to offer only two alternatives. The insects could travel toward light to which they are negatively phototactic in order to escape from the odor of menthol or toward the menthol in order to escape from the light. At 0.095 meter-candles 79 per cent of the flies were repelled by an air stream saturated with menthol; at 0.64 meter-candles, 74 per cent; at 2.58 meter-candles, 73 per cent; at 9.15 meter-candles, 69 per cent.

4. *Relation Between Physiological Action and Chemical Structure*

Most inquiries into the relation between stimulating effectiveness and chemical structure have assumed the form of field tests designed primarily to study attractance and repellence. These procedures introduced necessarily large experimental error in that many variables were beyond control. Not the least of these is the frequent occurrence of highly specific attractants. However, where field data can be translated into terms of acceptance and rejection thresholds, some interpretation of chemical and physiological interrelationships is possible. Such is true of the work of Cook (24).

In a study designed to ascertain the relation of physical properties of paraffin derivatives to the dilution most attractive to flies, Cook tested aliphatic alcohols from methyl to amyl and esters from acetates to valerates. To each of several small

flytraps he added a basic bait consisting of 25 ml. of 10 per cent molasses plus an aliquot of the alcohol or ester solution to be tested. The number of flies caught in each trap was taken as a measure of the attractiveness of the particular concentration contained therein. In this manner the optimum concentration of each compound was determined. It is a rough measure of the modal acceptance threshold.

Within each series of homologous compounds tested it was found that the acceptance threshold decreases with increased boiling point. A remarkably close correlation was observed A logarithmic plot of concentration and boiling point gives a straight line for which the approximate equation is: log. opt. conc. = 13.78 − 7.1 log. B. P. A comparable relationship exists between concentration and any property of the compounds which can be related thermodynamically to boiling point.

The relation between stimulating effectiveness and chemical properties can be examined in still another fashion. Instead of comparing the relative efficiencies of a homologous series by determining separately for each the most attractive concentration, the various compounds may be tested simultaneously in competition at optimum concentration. Since most of the compounds exhibit some repellence, the number of flies attracted is also a measure of the repellency. When the stimulative efficiency of each compound is expressed as the reciprocal of the number of flies trapped, the same general correspondence with boiling point as expressed above is observed. Increasing the chain length in a homologous series of aliphatic alcohols or esters brings about increased stimulative efficiency. This was first stated by Speyer (129). The addition of successive CH_2 groups to the acid side of the ester molecule results in a more pronounced and a more uniform increase in stimulative efficiency than when the additions are made to the alcohol portion of the molecule. This observation is borne out to a degree by similar studies of the codling moth by Eyer and Medler (46). Although in this case the addition of CH_2 groups to the acid portion of the molecule results in greater changes in physiological activity than similar additions to the alcohol side, the curve for the latter shows markedly fewer irregularities. It must be concluded that more exact experiments with the esters are required before any generalizations can be extended beyond the simple statement that stimulative efficiency is proportional to chain length, molecular weight and boiling point and inversely proportional to water solubility.

Several sets of the esters studied are isomeric. Cook stated that generally the isomer with the lowest acid radical attracts the greatest number of flies. In this series of experiments, however, there is extreme variation, and further experimentation is clearly indicated before data can be correlated. Especially is this true of those series where boiling points and solubilities of compounds with the same empirical formulae are very nearly identical. Differences between normal and iso compounds are more consistent. The normal compound possesses the greater stimulative efficiency as would be expected from its higher boiling point.

Many field experiments do not lend themselves to analyses such as the foregoing. The relative effectiveness of different members of homologous series can be determined only when the compounds are tested at comparable concentrations. Peterson's data (114), which indicate that saturated alcohols are attractive to onion maggot flies in the order iso-propyl, ethyl, butyl, amyl and methyl cannot be evaluated because the compounds were compared at concentrations which were not optimum.

Bunker and Hirschfelder (20), using mosquitoes, attempted to relate repellency to chemical structure but realized that the field technique employed introduced enormously high experimental error. Certain tendencies were suggested by the data. Ketones (e.g., acetophenone) appeared more stimulating than phenol esters (anisol) and than alcohols (camphor vs. borneol).

Studies of fly repellents (109) indicate that as a rule esters are more stimulating than their alcohols. This is to be expected in terms of boiling point, solubility, chain length and other physical properties.

5. *Humidity*

Whether or not responses to water vapor may be correctly characterized as chemoreception rests in the final analysis upon a fuller understanding of humidity reactions than is now enjoyed. Many aspects of hygrostimulation are so strikingly reminiscent of olfaction that water vapor cannot be denied the rôle of a chemical stimulus solely on the basis of its omnipresence in the environment.

The responses of insects to humidity may take the form of orientation to water vapor from a distance (80, 86, 87), proboscis responses to water vapor near at hand (86, 90, 138) and avoidance of regions of high or low humidities with consequent aggregation in a zone of preferred humidity. The response is frequently determined by the state of water balance of the insect. For example, the roach *Blatta orientalis* normally exhibits a preference for drier regions, but desiccation may reverse the reaction so that the animals become hygropositive (68). The behavior of the beetle *Ptinus tectus* Boie is dependent in a large measure on the degree to which H_2O is lost. Wet insects aggregate in a dry region until they become desiccated, whereupon the response is reversed (13). This is especially true of mosquitoes (133).

Only aggregation responses have been studied in great detail. Experiments usually involved observations of the reactions of insects placed in some type of ring gradient or alternative humidity chamber (69). In these arenas insects may show an intense response. Wireworm larvae, for example, entering a dry area, stop, move the head, manipulate the appendages, then retreat. Blood-fed female mosquitoes (*Culex fatigans*) show an equally decisive avoiding reaction when flying into an area of high humidity. In general, orientation involves klino-kinesis, klino-taxis, and ortho-kinesis (50, 69). No tropo-taxis or circus movements occur (50, 70).

With the African migratory locust (*Locusta migratoria migratorioides* R. and F.), which 'prefers' dry microclimates, the intensity of reaction to a humidity gradient as measured by the excess percentage ratio is related to the steepness of the gradient (85). With the mosquito *Culex fatigans*, which avoids high humidities (133), the mealworm beetle *Tenebrio molitor*, which avoids high humidities (117) and the wireworms *Agriotes obscurus* L. and *A. lineatus* L., which avoid low humidities (91), the intensity of reaction is related to the value of the highest humidity. The reaction of *Ptinus tectus*, which avoids high humidities, is most intense at low humidities. The discrimination factor varies in the same way. *Culex* distinguishes differences of one per cent RH near saturation while between RH 30 and 85 a difference of 50 per cent is necessary before it is appreciated (133). In general, the louse also shows greater sensitivity within the higher ranges of humidity (146). Wireworms can detect the difference between 100 per cent and 95.5 per cent RH. Lees (91) sug-

gested that the failure of reaction is due to an elimination of an integral part of the orientation mechanism under unfavorable physiological conditions rather than any real 'indifference' to humidity as such.

With all species studied the intensity of reaction to a given RH at different temperatures has been investigated. In this manner it has been found that the reactions of *Culex* and *Tenebrio* are more in accord with relative humidity than with saturation deficiencies. With wireworms, on the contrary, the intensity of reaction is more in accord with saturation deficiencies.

The behavior of species in the first category suggests that the sense organs are reacting as hygrometers to relative humidity. The reactions of wireworms suggest receptors of an evaporimeter type in that the intensity of reaction is dependent upon evaporation from some site on the body (91). This appears to be true also with the terrestrial isopod *Oniscus asellus* (139). In neither case, however, is the mode of action definitely understood. If receptors sensitive to RH are to act as hygrometers, the hygroscopic properties of the receptors determine their recording characteristics. Pielou found that curves relating RH to the hygroscopic properties of *Tenebrio* cuticle closely parallel curves relating RH to intensity of reaction. As he questioned, however, how can a receptor of this sort be affected more by the RH of the external environment than by that of the body fluids bathing its internal surface?

Various hypotheses have been advanced in an attempt to explain the mode of action of the evaporimeters. Pielou (116) postulated that evaporation could act by *a*) producing quantitative changes of chemical concentration, changes in osmotic pressure or mechanical stress, or *b*) by lowering the temperature, thus exciting heat receptors. Waloff (139) also suggested a concentration of body fluids to initiate reaction and as an alternative, secondary proprioception. Lees believed that the rapidity of reaction suggested local changes in osmotic pressure which might stimulate nerves directly. But none of these hypotheses is wholly satisfactory. Nor has positive identification of the receptors in some species illuminated the problem.

Humidity responses by *Tenebrio* are completely abolished when the antennae are amputated or covered with an impervious material (116). Two types of receptors are involved, pegs and pits. The former are confined to the distal seven segments. The latter are located on all segments except the terminal one. Removal of the terminal segments is followed by a decrease in the intensity of response, hence the peg organs are concerned in hygrostimulation. That they are not the sole receptors is proved when all seven segments bearing pegs are extirpated. The animals still respond. If eight pairs of segments are removed, response is abolished although the remaining segments bear pit sensilla. It has been shown by asymmetrical amputation that a threshold number of pit sensilla is necessary. An animal with the basal three pairs of segments (bearing pits only) intact does not respond to humidity changes. When a single segment on one antenna and five (two of which bear pegs) on the other are left intact, there is an 11 per cent response. This indicates that the pegs are humidity receptors but does not eliminate the pits since an animal thus treated bears both pits and pegs. When three segments are left intact on one antenna and five on the other, there is a 20 per cent response. The only difference between the two preparations is the greater number of pit sensilla, hence the con-

clusion that a greater number of pit sensilla is required. The peg receptors are also suspected of subserving the olfactory sense (137).

The humidity responses of wireworm larvae are abolished by the combined removal of antennae, maxillary palpi and labial palpi. No particular receptors have been incriminated and it has been concluded that responses are mediated by evaporation through these areas (91).

Wigglesworth (146) proved that the humidity receptors of the human louse are distinct from the olfactory receptors. Both are borne on the antennae. The former are tuft organs each of which consists of a minute cone bearing at its apex four tiny delicate hairs. The sensillum is innervated, like typical chemoreceptors, by a group of bipolar sense cells.

6. *Modalities and Discrimination*

Efforts to reduce the olfactory sensations of man to some system analogous to conceptions of taste modalities have been notably unsuccessful. It is hardly surprising, therefore, that few inquiries have been directed toward this aspect of chemoreception in insects.

Under the circumstances little more can be done than to study the reactions of insects to those odors which provoke distinctive sensations in man. This approach has been followed in a study of the reactions of honeybees to the odors of 47 essential oils and component compounds (56). Bees were trained to one odor against several empty odorless containers serving as controls. This odor was then exposed in the company of others and the frequency of visits to each recorded.

Of the 47 substances tested initially, three, in addition to the training odor were highly attractive. Each was an essential oil distilled from a citrus fruit and as such contained a motley array of chemicals. Common to all was limonene so that each emanated a similar odor. A tabulation of the number of visits to each indicated that this similarity of odor exists for bees also. Tests in which the four were exposed simultaneously indicated, however, that they were not indistinguishable. Comparisons of pairs of pure compounds showed that for the bee as well as man compounds with similar odors but different chemical structure were confused as one, while compounds with nearly identical structure but quite distinctive odors were easily distinguished. Of the first type nitrobenzene was tested with benzaldehyde and methyl ester of anthranilic acid with beta naphthol methyl ether. Counts of bees indicated that while the odors may have been very similar they were not completely indistinguishable. Man experiences like sensations.

If the olfactory sense of honeybees reflects similarities with that of man, it is to be expected that compounds with similar structure even to the point of stereoisomerism would evoke different sensations. This has been one of the obstacles to all attempts to relate smell to chemical structure. Thus within each of the following pairs the two members are clearly distinguishable by bee and man alike: amyl acetate and methyl heptenone, bromstyrol and phenylacetaldehyde, isobutyl benzoate and salicylic acid amyl ester, para-cresol methyl ether and meta-cresol methyl ether. It appears very likely, therefore, that whatever the mechanism of olfactory stimulation in bees, it resembles remarkably closely that of man.

Numerous theories which attempt to explain the nature of olfactory stimulation have been proposed and are reviewed briefly by Moncrieff (108). Recently Beck and Miles (10A, 98A) have advanced an infrared theory of olfaction, but complete details of their experiments have not yet been published.

CONTACT CHEMORECEPTION

1. *End Organs of Contact Chemoreception*

Contact chemoreception is treated below as a sense distinct from olfaction and the common chemical sense although in the present state of our knowledge, as pointed out above, we lack wholly satisfactory criteria for separating it from the former in the case of certain aquatic and subterranean insects. Localization of receptors and greater sensitivity afford a reasonable basis for distinguishing contact chemoreception from general reactions to irritants. Functionally, contact chemoreception as here limited is concerned principally and immediately with the acts of feeding and oviposition, and doubtless plays a rôle, often largely negative, in the choice of resting places.

Demonstrated loci of the receptors include: *a*) the *antennae* of bees (52, 84, 90, 97, 107) and of ants (126); *b*) the *mouthparts* or adjacent surfaces of the preoral cavity in many species (9, 32–37, 47, 49, 52–55, 60–63, 76, 86, 90, 98, 102, 106, 122, 125, 131, 138, 150); *c*) the distal segments of the *legs* in bees (52, 90, 97, 107); in flies (1, 8, 22, 27, 31–35, 42, 51, 55, 76, 79, 86, 102, 105); and in adult Lepidoptera (5, 99–101, 142); and *d*) the *ovipositor* of certain ichneumonids and braconids (40); and of *Gryllus*.

Verlaine (138) contended that the tarsal response of adult Lepidoptera is a reaction to humidity, viscosity and mechanical stimulation, dependent partly on associative memory; while McIndoo (95) ascribed reactions mediated by the tarsi of calliphorid flies to a combination of olfactory and mechanical stimuli: these views do not accord with the findings of the great majority of workers who have studied the tarsal chemical sense.

Phylogenetically it is of interest that ambulatory appendages have been identified as the seat of contact chemoreceptors in several other classes of aquatic and terrestrial arthropods (6, 12, 18, 78, 83, 92, 113); and in this connection it may also be pointed out that of the other loci demonstrated in insects, at least the mouthparts and the gonopods are presumed to have been derived from ancestral walking legs (128).

In most cases the nature of the actual receptors in insects is uncertain. Either the appendages, such as the antennae of bees, have a variety of end organs and subserve a variety of functions (tactile, olfactory, gustatory, hygroreceptive, thermoreceptive), so that attempts to match structure and specific function have been unsuccessful, or the regions concerned in contact chemoreception lack easily recognized end organs to which a chemoreceptive function could confidently be assigned. However, Minnich (103, 106) reported that he had succeeded in stimulating individually some of the long curved hairs on the aboral surface of the oral sucker of *Phormia* and *Calliphora* by direct contact with 1 M sucrose. In the earlier paper he said that "stimulation of the tip of a single hair may be sufficient for the response." This statement has been questioned by Frings and O'Neal (55), who quote Minnich's

later assertion that he moistened with sugar solution "all the hairs, marginal as well as central," and who therefore conclude that his experiment could not differentiate between the labellar setae and the numerous shorter hairs which lie among them. This later statement was not intended by Minnich to refer to experiments on the localization of receptors (personal communication). In their own examination of *Tabanus*, Frings and O'Neal were unable to obtain a response when only the longer marginal setae were moistened but found that contact of sugar solution with the tips of the thin-walled medium-sized hairs clothing the labella and lying among the longer hairs caused extension of the proboscis. Whether other types of sensilla present on the labella also may act as organs of taste could not be determined. Similarly, Frings and O'Neal showed that chemoreception by the legs of *Tabanus* is mediated either by medium-sized thin-walled hairs scattered among the long hairs which clothe the tarsus thickly, or by undiscovered organs on the integument beneath these hairs. The sensitive areas were the ventral surface of the distal half or two-thirds of the proximal tarsal segment and of the four more distal segments; the longer setae on these regions were insensitive. Dethier (36) observed that longer and shorter thin-walled spines are the only types of end organ common to the epipharyngeal and hypopharyngeal surfaces of the preoral cavity in larval Lepidoptera, and since these were the only areas which yielded a response to contact chemical stimulation he concluded that one or both types are among the specific receptors involved.

The above instances are the only ones known to the reviewers in which the effort to verify by direct experiment the gustatory nature of presumptive end organs has been even partially successful, and it is clear, as others have pointed out, that assignment of function on a morphological basis only, without experimental support, tends merely to obscure the situation. For this reason we are making no attempt at this time to reinterpret that rather extensive literature descriptive of the varied types of sensilla that are to be found on regions thought or known to be chemoreceptive on contact. The 'olfactory pores' (sensilla campaniformia) of McIndoo are now generally recognized as organs of proprioception (118, 119, 123, 124, 145), so that their presence on the tarsal segments of *Lucilia* and *Calliphora* (95) is probably beside the point. Eltringham (45) found on the tarsus of *Pyrameis* thin-walled hairs with multiple innervation; presumably these are the end organs of the function first investigated by Minnich (99), but the critical experiments which would prove that they rather than other undiscovered organs are the ones which respond to solutions have not been done. Hayes and Liu (77) have described from the tarsi of the housefly long, thin-walled setae, with a distinct joint at the base and with an innervation consisting of groups of spindle-shaped bipolar sensory cells; their inference that these are the end organs of contact chemoreception, though probably correct, is unsupported by experimental evidence other than observations (31–35) that *Musca* possesses a well-defined tarsal chemical sense.

Thus a major shortcoming of much of the work to date on contact chemoreception in insects has been the failure to identify positively the specific receptors involved. Since it is obvious that any attempt to comprehend the mechanisms concerned must take into account not only the characteristics of the stimulatory substances but also those of the receptors with which they interact, it is clear that progress in this field

will be limited until more definite knowledge of the structure and properties of the receptor elements is available.

2. *Methods of Investigation and Their Results*

Most of the physiological studies of contact chemoreception in insects have centered around the feeding response, which has been utilized in various ways by different workers as an indicator of chemical stimulation. By this means the location of the receptors has been roughly mapped out, many chemical compounds have been classified as acceptable or unacceptable and a considerable body of quantitative data has been developed in reference to the relationship between concentration and stimulatory effect. Further analysis of the categories acceptable and unacceptable has also been attempted with some success, and progress has begun toward a correlation of the physical and chemical properties of compounds with their potency in stimulation. A brief discussion of the more important methods that have been used in these studies will serve to indicate their limitations and provide a basis for a critical evaluation of the data which have been obtained.

a. FEEDING EXPERIMENTS; ACCEPTANCE AND REJECTION. The type of experiment most commonly employed leads to the determination of acceptance or rejection thresholds. For the former, an insect which has been allowed to drink until it no longer responds to plain water is presented with a series of dilutions of the test chemical, and the lowest concentration which induces an attempt to feed is noted. In the measurement of rejection thresholds, the test compounds may be given to thirsty insects in water, but since it is difficult to regulate the degree of thirst it is often more convenient first to let the specimens satisfy their desire for water and then to offer dilutions of the test compound in a standard solution of some acceptable substance, such as sucrose. In either case, the least concentration which prevents acceptance or causes an avoiding reaction is taken as the threshold of rejection. Obviously such measurements can have only a relative value, and the results are dependent in part on the strength of the attractive stimulus.

b. FACTORS WHICH AFFECT THE RESPONSE. Responses of acceptance or rejection, separated as they are from the initial process of sensory excitation by a complex chain of physiological events and subject therefore to modification at various levels by the physiological state of the animal, are open to influence by a variety of factors which must be suitably controlled if consistent results are to be obtained. Among these have been noted: previous association (conditioning), preceding chemical stimulation, availability of natural sources of nectar or other food, age, preparation for experiment and especially the condition of the animal in regard to nutrition and thirst.

Conditioned responses. Several workers have succeeded in conditioning insects to respond positively to one of a chosen pair of chemicals and to reject the other (9, 84, 122, 125, 126). In repeated trials the animal learns to associate one stimulus with the presence of food, with which it is rewarded after a positive response, and the other with some disagreeable experience, such as the taste of food heavily contaminated with quinine. The ability of aquatic beetles to distinguish between NaCl and HCl, NaCl and sucrose, HCl and sucrose, etc., was demonstrated in this way; and ants

were trained to respond positively to 0.5 M sucrose and negatively to water. Theoretically it should be possible with this technique to determine specific thresholds for the test substances, but the few studies of contact chemoreception in which the method was used were made under conditions which usually did not permit a very accurate estimate of the strength of the chemical stimuli employed. Associations thus formed seldom persisted more than a few hours without reinforcement, yet the fact that they were established readily on repeated occasions suggests that care should be taken in routine measurements of acceptance and rejection thresholds to avoid complicating the results by introducing factors of this sort.

Preceding chemical stimulation; adaptation. Chemical stimulation which precedes a given test by a sufficiently short interval also may influence the results, but in a manner somewhat different from the learning process just described. Probably several distinct mechanisms are involved. Frings (54), for example, noted what he termed a 'shock reaction' in roaches exposed to supraliminal concentrations of acid: after contact with such a solution, they often refused everything, even sucrose, for a time. Similar behavior was seen in houseflies after exposure of the tarsi to five per cent $HgCl_2$ in 1.0 M sucrose (31, 33). One hundred and twenty flies were tested, and immediately after exposure only one fly would respond to sucrose. Recovery was gradual, with 15.8 per cent response after one hour, 46.4 per cent after three hours and 68.0 per cent after five hours. It is possible that the receptors were injured temporarily in these experiments. Another type of reaction was demonstrated by Minnich (105). When testing was begun below threshold and flies were transferred in immediate succession from one sugar solution to another in increasing order of concentration, individuals whose threshold for sucrose was ordinarily 1/1600 M did not respond until a concentration of 2/100, 4/100 or even 8/100 M was reached. This result was ascribed to fatigue or adaptation. Others (90, 138, 142) have recorded similar observations, which they also interpreted as evidence of fatigue or adaptation of the receptors, but in these cases the differences measured are hardly significant statistically. Von Frisch (63) noted that weaker solutions of sucrose were taken hesitantly or refused by bees for the first 5 or 10 minutes after stronger ones had been fed, though he states also that the acceptance threshold (0.25 to 0.125 M) was not changed even after 0.5 M sucrose had been fed for hours nor when the threshold was approached gradually from above. Other workers (44, 97) could find no difference in the threshold of acceptance determined when sugar solutions were offered in ascending or descending order of concentration. Thus, while it seems probable that sensory adaptation does occur, the evidence which has been gathered is for the most part weak or contradictory, and all of it is based on responses contributed to by an extrasensory element which it is difficult to assess. The observations suggest that, if adaptation does take place, recovery is fairly rapid; failure to demonstrate it more conclusively may have resulted in some cases from too long an interval between tests. Further study of the phenomenon, in experiments designed to separate central and peripheral factors, would be desirable.

Availability of natural food. Both von Frisch (60, 63) and Kunze (89, 90) have remarked that the threshold of bees for sucrose and other sugars is dependent to some extent on the availability of nectar and the sugar concentration of the latter. At

seasons when nectar with a high concentration of sugar is in good supply, bees will refuse sugar water in dilutions which are accepted readily when natural sources are less favorable, even though under the latter conditions they are kept equally well fed and have a plentiful store of sugar or honey in the hive. The mechanism whereby the response is modified is obscure but does not seem to involve either sensory adaptation or the state of nutrition.

Age. No thorough investigations have been made of the effect of age on the contact chemoreceptive response. In the extensive observations of von Frisch (63) little influence of age on threshold was seen until the workers had been gathering nectar for about a month. These elderly bees had an elevated acceptance threshold for sucrose. Eger (44) found that fifth instar larvae of *Acidalia* had higher thresholds of rejection for NaCl, HCl and quinine than younger specimens, but Dethier (36) noted little change in thresholds with age among other caterpillars, except for a general disinclination to feed immediately before pupation. In wireworms, orientation to chemical stimuli shows a biennial rhythm, of which the biting response is independent (136). Probably age is of minor significance in most investigations of contact chemoreception apart from cases where marked changes in dietary behavior are associated with the process of development.

Preparation of specimens. The practice of mounting insects for experiment, introduced by Minnich (99) and since used by many other workers, often renders the specimens erratic or unresponsive for the first 24 hours or so after preparation. Later this inconsistency disappears, and with suitable treatment the insects may be maintained in a responsive state for weeks or months.

Nutrition. By far the most important factor affecting acceptance thresholds is the nutritive state of the animal, and it is a common observation that acceptance thresholds fall when insects are maintained continuously on a water diet or are otherwise subjected to complete or partial starvation (5, 27, 34, 35, 60–63, 76, 79, 89, 101, 102, 105, 136, etc.) Hunger may reverse the effects of training and cause an insect to accept quinine-flavored meat which he has been taught to avoid (125). However, a marked decrease in threshold with starvation does not invariably occur (5, 37, 55, 63, 106, 126), and the time course and extent of the decrease may be quite different for different compounds (76, 105) or for oral and tarsal receptors (76). Where rejection thresholds are determined against the background of a contrasting acceptable stimulus, an increase in the rejection threshold may be expected with starvation if the latter results in increased acceptability of the contrasting stimulus (150). Thus, lack of water has been shown frequently to be a potent force in improving the acceptance of otherwise repugnant solutions, and it is known that, even apart from starvation or thirst, variations in the strength of the attractive stimulus do produce differences in the rejection thresholds determined (33, 34, 44, 63, 138). Haslinger (76), in recognition of this effect, measured the rejection threshold for HCl of blowflies during starvation by presenting the acid in a fructose solution the concentration of which was varied so as to be just three times the threshold for fructose on each day of the test. Under these conditions, no change in the rejection threshold for acid was observed, and similar results were obtained with unacceptable sugar alcohols, salts and quinine. However, von Frisch (63), who presented NaCl, HCl and quinine in 1.0 M sucrose to bees at all stages of starvation, found no change in

the rejection threshold for salt and an increase with HCl and quinine. Bees succumb to starvation so rapidly that they are not the animal of choice for experiments of this sort (15–17, 63). Acceptance of $HgCl_2$ in 1.0 M sucrose by *Phormia regina* was better after 48 hours than after only 24 hours on a water diet (34). Frings and O'Neal (55) observed somewhat variable rejection thresholds in *Tabanus* during starvation, but no consistent trend appeared in their data. In summary: there is usually but not always a decrease in acceptance thresholds during starvation; the data in respect to rejection thresholds are scantier and to some extent conflicting so that more study is needed.

The contention of various authors, that the lowest threshold of acceptance reached during the penultimate stages of starvation represents a threshold of receptivity or a specific threshold, requires examination. The state of starvation is conceived as eliminating a central inhibition which under normal circumstances prevents a visible response to low, though stimulating, concentrations of the acceptable compound (105, 106, 136). With bees, a rather constant value for the acceptance threshold of sucrose is always obtained during starvation, irrespective of initial differences, and it is considered unlikely that a starving insect would refuse an acceptable substance that it was capable of perceiving (63). A weakness of these arguments, apart from their speculative nature, lies in the fact that many variables other than hunger may be operating to condition the central nervous state: although starvation may have lowered the resistance, so to speak, between excitation and acceptance, there is no guarantee that the resistance has been reduced to zero. Granted, however, that the premise is correct, the threshold measured can be only a specific threshold and not an absolute threshold. From experiments with man (e.g., 82) it has long been known that the specific threshold for a given compound need not be the absolute threshold of excitation, since it is often possible for human subjects to distinguish dilute solutions from distilled water at concentrations so low that the solute cannot be identified nor even classified in one of the usual taste categories. Whether a similar situation exists for insects is uncertain, but that the sensory process may be at least as complex as in the vertebrates is shown, for example, by the following experiment of Frings (54) with the American roach: on adding small amounts of acid to 0.1 M sucrose, a concentration was presently found which caused hesitation and rejection. A further increase of acid brought the solution into a range in which it was accepted more readily than sucrose solution, while a still further increase disclosed a threshold above which all concentrations were rejected vigorously. This level was interpreted as the rejection threshold. In this experiment it is clear that the acid was in some manner stimulating the roach at a concentration below either the acceptance or rejection threshold, and it is possible that the reaction to very low concentrations represents a process similar to the phenomenon of a general or difference threshold in man.

c. VARIABLES WITH LESS PRONOUNCED EFFECTS. Somewhat surprising are the observations that the factors of light intensity, temperature, humidity and sex, in contrast with their effects on olfaction, and the presence or absence of odors, appear in general to have little effect upon the sensitivity of insects to chemical contact with solutions in laboratory investigations, although it is well known that these may exert profound effects upon the feeding behavior of insects in nature.

Light intensity. Only casual observations of variations in light intensity during

tests have been recorded, and any effect which they may have had has been overshadowed by other variables. Kunze (90) states that bees whose eyes were covered with opaque shellac responded no differently than normals.

Temperature and humidity. Weis (142) obtained essentially identical distributions of acceptance thresholds for sucrose in tests run with butterflies at 25.5° C., 56.7 per cent RH and at 35° C., 100 per cent RH. Nor was the threshold altered when the butterflies were kept at 21° C. and the sugar solutions offered at 15° C., 21°C. and 35° C. For bees, von Frisch (63) found temperature changes within wide limits to be without significant effect on the acceptance ratio, both in the field where the temperature of the bees followed the ambient temperature and in laboratory tests where the temperature of the solutions was varied independently over the range from 10° to 35° C. Yet the amount of sugar solution drunk by bees is greater at higher temperatures. In another group of studies with bees at air temperature (20° to 25° C.), von Frisch observed no change in acceptance of 1.0 M sucrose within the range from 8° to 50° C., but the majority of specimens became hesitant when the solutions were at 3° or 5° and at 55°, while general refusal occurred at 1° and 60° C. These experiments give incidental added proof that viscosity is not a significant factor in insect chemoreception. The apparent absence of a temperature effect in insect gustation is in contrast with results which have been obtained with man, but it may be remarked that there has been little agreement among the numerous investigators who have studied human taste from this point of view. References to many of the studies on man may be found in the papers of Goudriaan (65) and Hahn and Günther (71).

Sex. No difference in the sensitivity of male and female specimens appeared in the studies of Abbott (1) with *Cochliomyia macellaria*, of Deonier (34) with *C. americana* and *Phormia regina* nor in our own tests with the latter, of Minnich (105) with *Calliphora vomitoria*, of Anderson (5) with various butterflies, nor of Frings (54) with the American roach. Drone bees responded more readily to 1.0 M sucrose than workers, while no difference was detected in the response of young queens and workers of the same age (90).

Odors. The acceptance of sucrose by bees was not affected by the presence or absence of the odors of essential oils or honey, and removal of the olfactory organs by antennectomy left the taste threshold unaltered (63). Amputation of antennae, labial palpi and the forelegs of nymphalid butterflies did not significantly affect the tarsal response to solutions (99). The average tarsal acceptance threshold of 181 specimens of *Phormia* for sucrose was 0.019 M ± 0.005 (standard deviation); for 122 of these same flies which survived combined antennectomy and labellectomy it was 0.017 M ± 0.013. After operation the flies no longer responded to odors (42). Ritter (122) found that amputation of the antennae was without effect on the gustatory response of *Hydrous;* however, this beetle has olfactory receptors on the maxillary palpi. Bauer (9), who worked with the same species and with *Dytiscus*, states that removal of both the antennae and the maxillary palpi, which abolished the olfactory response, did not prevent a response to taste substances, but that the threshold was raised and the animals were generally more sluggish. The increase in threshold in this case may be ascribed to loss of part of the contact chemoreceptors, some

of which are located on the maxillary palpi, to a deficient central excitatory state, or to both factors.

d. EXTENSIONS OF THE FEEDING TECHNIQUE. *Method of mixtures.* While many of the facts already cited have come from work with conditioned insects or from straightforward feeding experiments, certain variations of the latter technique have been made to yield additional information not otherwise obtainable. One such refinement was developed by von Frisch (62, 63), who noted that the stimulative effect of various sugars for the bee is additive. For example, a mixture of sucrose and fructose, each present in a concentration which would have been refused if offered alone, was accepted readily. It occurred to von Frisch that this fact could be utilized in retesting the acceptability of sugars which had appeared to be without stimulative power even in saturated solution when presented singly. Such sugars were now mixed with a concentration of sucrose to which some but not all of the bees would respond, and it was found in fact that the acceptance ratio was improved in several instances. By comparing the concentration of the second sugar with the concentration of sucrose which had to be added to produce an equal effect, a quantitative estimate of the relative 'sweetness' of the second sugar was obtained. In similar experiments, von Frisch determined the stimulating power of various compounds repugnant to the bee by adding them in known quantities to an acceptable solution of sucrose and observing the decrease in acceptability. This latter procedure illustrates the usual approach in the measurement of rejection thresholds.

Measurement of 'crop loads'. Another variation of the feeding technique, also due to von Frisch (63), is based on the observation that the quantity of sugar solution taken by a bee at one feeding is a function of the concentration. At 0.5 M, the average bee drank 44.9 cu. mm. per trial, but when 1.0 M or 2.0 M sucrose was offered the amount taken rose to 50.5 and 55.2 cu. mm., respectively. Thus a difference in the degree of response was revealed beyond that indicated by a simple count of acceptances and rejections. Having provided a concentration of sucrose just sufficient to ensure acceptance by all the bees in the test group, von Frisch then showed that addition of NaCl in concentrations too low to reduce the percentage accepting still produced a measurable decrease in the average amount of solution drunk per trial per bee. This was interpreted as an indication that the bees perceived the presence of the salt even though they drank the solution containing it; in other words, that the specific threshold for NaCl must lie well below the rejection threshold. If this conclusion is acceptable, the technique offers an additional means of approximating specific thresholds, and of testing intensity discrimination.

The same procedure has also been used by von Frisch to draw a distinction between various types of compounds unacceptable to the bee. When concentrations of quinine hydrochloride below the rejection threshold were added to sucrose solution, the outcome differed from that obtained with NaCl in that no decrease occurred in the quantity of solution taken; and this, von Frisch reasons, shows that there is a difference in the quality of the perception of the bee for these substances. HCl was found to behave in this respect like NaCl, but by no means all of the bitter substances tested gave results similar to quinine. Von Frisch concludes that what is bitter for man may not constitute a homogeneous grouping for the bee.

Duration of feeding as an index of acceptability. A somewhat similar modification of the acceptance technique was used in conjunction with the usual procedure by Weis (142), who recorded the length of time that butterflies, stimulated tarsally, would leave the proboscis in an acceptable solution. With *Pyrameis atalanta* Linn. as the test animal, she observed that the proboscis remained in distilled water for 25 seconds on the average, while the average drink of 0.5 M sucrose required 4 minutes 19 seconds. Dulcin (*p*-phenetyl urea), a synthetic sweetening agent, caused withdrawal of the proboscis after only eight seconds, and was therefore judged to be somewhat distasteful to the insects. Without further investigation, too much reliance should not be placed on the use of this method with Lepidoptera in view of our ignorance of the process of chemoreceptive adaptation in insects and of Stober's (130) finding, incidental to a study of digestive function, that several species drank indiscriminately the following: various concentrations of sugar solution, with or without added dyestuffs; water; litmus; congo red; neutral red; brom cresol purple; brom thymol blue; milk; frog or rabbit blood, with or without Na-oxalate or hirudin; alcohol; a variety of salts and acids; starch; water colors; Flemming's fixative; and Schaudinn's solution. It is true, of course, that in Stober's experiments the tarsi were not in contact with the fluids as they were in the experiments of Weis, but Weis herself points out that duration of feeding is not a reliable criterion of acceptability if the animals are starved and thirsty. Kunze (89) likewise used duration of feeding as an index of acceptability of solutions for the bee.

Tarsal stimulation. One of the most fruitful modifications of the food acceptance technique was introduced by Minnich (99) consequent upon his discovery that contact chemoreceptors are present upon the tarsi of certain nymphalid butterflies and that stimulation by an acceptable substance results in extension of the proboscis. The location of these sense organs makes it a simple matter to investigate their function without giving the animal an opportunity to ingest the test substances, a fact which facilitates control of the important variable of nutrition and which permits one to avoid some of the complications which arise when toxic compounds are investigated. Further observations by Minnich (102, 107) demonstrated similar sensitivity to chemical stimulation in the legs of flies and bees. Since then both he and others have taken advantage of this convenient arrangement on numerous occasions (see references under section on location of receptors above). The present writers recently combined the technique with extirpation of the olfactory receptors in flies, a procedure which permits testing of water-soluble organic compounds whose odors might otherwise cause confusion in the results (42). A method which would allow similar tests with substances which are insoluble in water would be highly desirable as this would extend greatly the range of compounds whose effects on contact chemoreceptors could be investigated. The principal problem presented here is the choice of a suitable diluent, which must combine the following characteristics:

1. It must be a solvent for the compounds to be tested and must not react chemically with them.

2. Preferably it should be nonstimulating; if stimulating, then preferably attractive; if inert or repellent, then it must dissolve some standard attractant, such as sucrose, in sufficient concentration to ensure a positive response.

3. It must lack high contact toxicity.

An attempt in this direction, using ethylene glycol as a carrier for the attractant (sucrose) and for the test substances, has been only moderately successful.

Ovipositor preparations. One further technique which offers considerable promise developed from the observation that the ovipositors of certain insects respond to contact chemical stimuli (40). It has been found that the isolated abdomen of some parasitic hymenoptera makes characteristic avoiding movements if the valvulae are placed in solutions which are rejected when presented to the antennae, mouthparts or tarsi of other insects. Rejection thresholds for such compounds can therefore be determined with this preparation, which not only eliminates the nutritional variable of other techniques, but also renders unnecessary the addition of an attractant to the test solution. Abdomens protected from desiccation by coating the cut surface of the petiole with paraffin remain responsive for several days if kept in a moist atmosphere at room temperature. Similar preparations may be made from the cricket *Gryllus*, and probably from some other insects.

Electrical recording from chemoreceptors. To have some means of studying receptor function more directly than via the initiation or suppression of reflex responses would have many advantages. Recording of sensory action potentials offers a possibility, but so far all efforts along these lines with the chemoreceptors of insects have been fruitless. Dethier and Prosser tried in vain to record chemically stimulated action potentials from the antennal nerve of caterpillars, while Dethier and Roeder obtained only negative results with preparations of the nerves which supply the ovipositor of parasitic wasps and the cricket, and the mouthparts of the roach (unpublished experiments). The individual sensory fibers are of small diameter; the presumably small potentials are apparently shunted out by adjacent tissue and fail to rise above the noise level in the amplifying system. In these unsuccessful trials all the preparations responded with visible movements to chemical stimulation, so that there can be no doubt that normal receptor function was maintained; and tactile stimulation of the regions concerned produced typical spikes in the oscillograph records (38). Additional attempts of this sort should be made whenever favorable preparations are encountered, in spite of the technical difficulties imposed by the small diameter and short length of the nerves, for they offer the best prospect of obtaining information uncomplicated by central nervous factors about the chemical response in insects. The fact that some success has attended similar efforts in vertebrates (3, 7, 75, 81, 115, 120, 154) should encourage further endeavor.

3. *Sensitivity in Taste*

a. SPECIFIC DIFFERENCES. Conspicuous differences in threshold are found even among closely related insect species (cf., e.g., 5, 44, 126), and consequently it is hardly possible to generalize in comparing the acuity of the insect gustatory sense with that of man and other animals. The known range of concentrations adequate for stimulation lies approximately between 12 M and 1×10^{-6} M, depending on the species, the individual, the organ, and the substance concerned. Limitations of space prevent the citation of more than the following representative figures for commonly tested compounds (see table 2, page 244).

b. INDIVIDUAL DIFFERENCES. As nearly all workers have noted, different individuals of the same species show a wide range of thresholds for a given compound, in spite of all precautions taken by the experimenter. Von Frisch (63) even gives evidence that the sensitivity of the same bee may vary from day to day. These variations pose difficulties in the comparison of data and in the presentation of results, which lose much of their significance unless accompanied by some measure of their reliability. In this connection it is useful to know that the scattering of thresholds in a population of *Phormia* reared under standard conditions is not significantly different from a normal distribution when plotted against the logarithm of concentration (42), and that a similar relationship may be demonstrated for both acceptance and rejection thresholds of other insects in most cases where sufficient data have been reported (e.g., 44, 54, 63, 142). It is therefore possible to characterize the reaction

TABLE 2. COMPARISON OF TASTE THRESHOLDS

COMPOUND	THRESHOLD CONCENTRATIONS[1]
sucrose	man, 0.02 M; bee, 0.06–0.125 M; butterfly (*Pyrameis*), average ca. 0.01 M, in starvation as little as 8×10^{-5} M, or for *Danaus*, 9.8×10^{-6} M; horsefly (*Tabanus*), 0.005–0.11 M.
NaCl	man, 0.009 M; bee, rejects ca. 0.24 M in 0.5 M sucrose; various caterpillars reject at 0.2 M, while others accept over the full range up to and including 5.0 M.
HCl	man, 0.00125 M; bee, rejects 0.001 M in 1.0 M sucrose; various caterpillars reject at 0.01–0.2 M.
quinine	man, 1.5×10^{-7} M; bee, rejects at 8×10^{-4} M in 1.0 M sucrose; various caterpillars reject at 0.002–0.033 M; aquatic beetles were conditioned to respond to 1.25×10^{-6} M.

[1] Data on man are for specific thresholds, adapted from (108); for the bee, from (63); for *Pyrameis*, from (101); for *Danaus*, from (5); for *Tabanus*, from (55); for caterpillars, from (44); for aquatic beetles, from (9). The figures for insects are thresholds of response.

of a population to a given chemical mathematically in terms of the regression of responses, expressed in probability units, on log concentration, and to calculate within stated limits of accuracy the most probable value of the concentration required to produce a chosen degree of response. While one might be tempted to regard the logarithmic nature of this distribution as evidence for the operation of Fechner's rule in insect chemoreception, it should be kept in mind that the relationship describes the distribution of thresholds in a population of individuals, each of whom bears a population of receptors, and that there is at present no information as to the manner in which the number of receptors responding or the response of a single receptor element depends on the intensity of the stimulus.

However, information obtained with other animals suggests that the phenomenon is probably more widespread in chemoreception than is generally realized. Rather similar to the results with insects are the data of Krinner (88), who trained the min-

now, *Phoxinus*, to distinguish sucrose or salt from water and from each other, and who noted that the intensity of reaction (number of responses) was roughly proportional to the logarithm of concentration. Crozier (29, 30) demonstrated a direct proportionality between the logarithms of speed of reaction and concentration of the stimulating compounds with worms exposed to acid and alkaline solutions. In the rabbit, Hasama (75) found an approximately logarithmic relationship between the strength of the negative electrical potential developed on the hippocampal lobe and the intensity of the odor of quinoline, while Hahn and Günther (71), in experiments on the temperature dependence of human taste thresholds, remarked that the range of variation was approximately proportional to the concentration. Lemberger (91A) demonstrated that the discrimination factor in human taste is constant for sucrose and Na-saccharin, except at the extremes of concentration. It is thus apparent that the factor of stimulus intensity enters logarithmically into a number of the phenomena associated with chemoreception although the relationship has been investigated less thoroughly here than with some of the other senses.

c. *Sensitivity differences of different organs.* Even in a single individual the threshold for a given substance may be different if different organs are stimulated (32, 34, 55, 76, 97, 102, 105, 106, 122). Thus, in *Tabanus*, the average labellar threshold for sucrose was 0.021 M; for the tarsi, it was 0.060 M (55). With most of the compounds tested on *Calliphora erythrocephala* by Haslinger (76), the labella were more sensitive than the tarsi; but in *C. vomitoria*, according to Minnich (106), the oral lobes are only one-sixteenth as sensitive to sucrose as the legs although the fly reacts to lactose applied orally but not when it is touched to the tarsi (105). Houseflies would extend the proboscis after contact of the tarsi with 1.0 M sucrose which contained a toxicant in suitable dilution but avoided the fluid or refused to drink it when it touched the mouthparts (32); and the same was observed with *Cochliomyia americana* and *Phormia regina* (34). Rather similar observations were reported by Verlaine (138) for *Pieris*. The antennae of the bee are more sensitive to solutions than are the legs (52, 97, 107), and the proboscis more sensitive than the antennae (90). According to Ritter (122) the beetle, *Hydrous*, can detect HCl at a concentration of 0.007 M with the tips of the labial palpi; these are said to be insensitive to NaCl and sugar, which are perceived via the maxillary palpi. An interesting problem which should repay future investigation is the extent to which threshold differences of different organs in the same insect are dependent on receptor sensitivity, number of receptor units activated and central nervous factors. As yet there has been no productive research on the possibilities of temporal and spatial summation, nor is there any information on latency or other temporal aspects of receptor function.

d. *Intensity discrimination.* Only an approximate estimate is available of the ability of the individual insect to discriminate between slightly different concentrations of the same chemical. Von Frisch (63) showed that there were measurable differences in the response of a group of bees to 1/8 M and 5/32 M, or to 1/4 M and 5/16 M sucrose, while no changes in the response were detectable by his methods when the concentration intervals were half as great. In other cases where geometric series have been used, the least ratio between successive concentrations appears also to have been 1:1.25 (54, 55); thus we know that, like the bee, the American roach and

the horsefly, *Tabanus*, can distinguish at threshold between solutions which differ by no more than this factor, but the lower limit of their discriminatory ability has not been explored. Somewhat greater sensitivity was claimed by Verlaine (138) for the oral receptors of *Pieris* on the basis of tests with two specimens. No technique has been devised which would measure the discrimination factor in insects at concentrations above threshold. According to Moncrieff (108) the discrimination factor for the chemical senses of man is about 0.3, i.e., nearly the same as for the insects mentioned above, but Lemberger's data for human taste indicate values of about 0.15 for sucrose and 0.11 for Na-saccharin, except at threshold and at very high concentrations (91A).

4. *The Modalities of Taste in Insects*

It is generally believed that man distinguishes four types of taste sensation, viz., sweet, salt, sour and bitter, and that most complex tastes can be resolved into some combination of these four primary qualities (14, 108). In view of the work of Allen and Weinberg (see also the study by Pfaffmann, 115), this interpretation, criticized vigorously by Henning (77A), probably represents an oversimplification of the actual state of affairs, although their measurements of the critical frequency of electrical stimulation did reveal four distinct sets of receptors, each of which it was possible to identify chiefly with one of the primary sensations (4).

In insects all that has been observed directly is that some substances are accepted and others rejected, and it has been necessary to resort to less straightforward methods in the attempt to ascertain whether the categories acceptable and unacceptable are homogeneous or whether insects also are capable of a finer degree of discrimination. Recent evidence has been derived principally from five sources: *a*) observations of the behavior of specimens conditioned to respond to a given chemical when confronted with other compounds; *b*) determinations of the additive or non-additive capacity of diverse chemical stimuli; *c*) measurement of the amount of sucrose solution drunk by bees after contamination with subliminal concentrations of different chemicals: *d*) comparison of the threshold changes for different compounds during starvation; and *e*) localization of the receptors for certain types of substances on definite organs.

Bauer (9) trained *Dytiscus marginalis* and *Hydrous piceus* to respond positively to sucrose; these animals could not be trained simultaneously to avoid glucose, and also reacted positively in the majority of cases to some 15 or 20 other sugars and sugar derivatives. As in the experiments of Schaller (125) and Ritter (122) with the same or related species, the beetles learned readily to distinguish between pairs chosen from sucrose, NaCl, acids (Hcl and HAc) and quinine, and could even be trained to accept quinine and avoid NaCl. But specimens which had learned to avoid HCl also avoided HAc and could not be taught to respond differently to the two; the same result was obtained when quinine was matched against salicin or aloin. Bauer concluded that the sweet substances (with the possible exception of mannose, which was avoided by some individuals) constitute a single homogeneous grouping; salt, acids and bitter substances, normally avoided, were distinguished from sweet and from each other, so that taste substances could be classified into the same four qualities for these beetles as for man.

As mentioned above in the discussion of methods, von Frisch (63) found additive the stimulatory effects of all sugars which are acceptable to the bee. Summation was noted also between NaCl and LiBr, NH_4Br or HCl, but the repellent effect of quinine was lessened rather than enhanced by the addition of acids (hydrochloric, acetic, sulfuric, citric, lactic). When sucrose solutions, one containing NaCl and another quinine hydrochloride, were prepared which were accepted by equal proportions of the bees, they were drunk in different amounts. Almost as much of the quinine was taken as of the control, but considerably less of the solution containing salt. Thus far, the results would indicate three taste qualities: sweet, acid-salt and bitter. But in other experiments von Frisch found that starved bees show no better acceptance of NaCl than when fully fed, although the threshold for rejection of quinine rises eight times and that for HCl by a factor of five. Therefore, he concluded that salt represents a quality different from either acid or bitter. Furthermore, although there is no summation of repellency between quinine and acids, other bitter substances, such as aloin, arbutin, colocynthin and salicin, are rendered more repugnant by the addition of HCl, so that the category bitter is not homogeneous for the bee. It is possible that investigations of this kind would reveal similarly complex relationships in man.

Other evidence as to the separation of the taste qualities in insects was provided by Ritter (122), who found that after amputation of the maxillary palpi specimens of *Hydrous piceus* would still react to 0.007 M HCl but not to salt, sugar or quinine. Removal of the tips of the labial palpi then abolished the response to acid although animals lacking both sets of palpi and the antennae still reacted positively to meat juice, presumably via receptors in the mouth. These observations would seem to set the acids apart from other taste substances for *Hydrous* but require reinvestigation in view of Bauer's (9) finding with the same and related species that amputation of the maxillary palpi left the gustatory response to sucrose, etc., unaltered except for an increase in threshold. It is unfortunate that Bauer failed to comment on this discrepancy between his results and those of Ritter.

Several of the observations above are rather difficult to reconcile with the hypothesis put forward tentatively by Frings (54) that all specific tastes may be merely points in a continuous spectrum of taste sensation, the critical dimension of which is envisioned as penetrating power or some colligative property. While it is true that in general sweet substances are those which penetrate cells slowly, that some salts and alkalis at low concentrations are acceptable to insects and sweet for man, that there is a gradual transition from typical salty to bitter in the series of inorganic salts as their ionic mobilities increase, and that the most mobile cation, H^+, is the principal factor in the sour taste, such facts as summation of certain types of chemical stimuli but not of others, the apparent grouping by conditioned insects of chemically similar compounds or of those which taste alike to man and the sharp localization of acid reception alleged to occur in *Hydrous* do not fit easily into the scheme proposed. On the other hand, the experiments of Allen and Weinberg (4) reveal a high degree of interdependence among the four receptor types in man and thus raise a question as to whether the concept of a pure primary taste sensation may not be an abstraction (cf. Henning, 77A). There are also anomalies in human taste, such as the sensations aroused by alkalis, borax, hydrogen peroxide, ginger, menthol and some

metallic salts, not all of which are readily assigned to one or another combination of the recognized primary qualities (Moncrieff, 108). The invocation of thermoreceptors, tactile receptors and the common chemical sense to explain such aberrant phenomena is not wholly convincing in the absence of experimental evidence that they are actually concerned. In the cat, Pfaffmann (115) was able to distinguish only three types of taste fibers. Individual fibers of one type responded to acid stimuli (and to KCl); of another to acids, NaCl, NaAc, KCl, and $CaCl_2$; and of a third to acids and quinine. Among the several students of vertebrate gustation who have used electrical methods, only Zotterman (154) succeeded in obtaining a single fiber response to stimulation with sugar solutions, and this in only one instance. This suggests that most of the nerves which mediate the sensation 'sweet' in the vertebrates, like the chemoreceptive fibers of insects, may give rise to potentials too small for recording with present methods. The implication of the other observations above is that the entire question of the physiological basis for the modalities of taste stands in need of much fuller investigation than it has yet received, in man as in the insects, so that an hypothesis such as that of Frings may yet have value, even if it is only that it will stimulate work along these lines. Among the insects, further experiments with conditioned responses and more studies on the interaction of diverse stimuli should be productive pending the development of methods for a more direct attack on receptor function.

5. *Stimulation in Relation to Chemical and Physical Properties*

According to available data about 310[1] compounds have been presented experimentally to the contact chemoreceptors of insects. Of these, some 46 are sugars or sugar derivatives, 31 of which have been found acceptable to one or another species. With few exceptions the remainder, which include unacceptable sugars and related compounds, alcohols, aldehydes, alkaloids, amides, amines, amino acids, bromo- and chlorohydrins, carbitols, cellosolves, esters, ethers, fats and oils, fatty and mineral acids and their salts, glycols, glycosides, ketones and proteins, plus a few miscellaneous cyclic compounds and synthetic sweetening agents, are all rejected actively if presented in sufficient concentration. Of this array, the acids and salts, at low concentration, are sometimes taken in preference to plain water or dilute solutions of sucrose (cf., e.g., Butler, 21; Frings, 54), so that they alone exhibit a double modality, being acceptable in one range of dilutions and rejected at higher concentrations. Several fats, oils and proteins release a biting response in wireworms (136) and would doubtless be acceptable to various other insects. Exceptional behavior toward certain compounds is occasionally encountered. Thus, saturated solutions of NaCl were accepted by some butterflies (142) and by *Calliphora erythrocephala* (76), as by certain species of caterpillars (44). *Cecropia* larvae took strychnine freely when it was placed on the leaves of their food plant (53), and there are of course many similar examples of the ingestion of toxicants, not considered here, to be found in the literature on insect control.

[1] This estimate excludes the several thousand organic substances which were screened as potential insecticides and repellents by various agencies, principally the U. S. Department of Agriculture, during the war. A full report on these tests has not yet been made public.

Concerning the manner in which chemoreception in insects is related to the physical and chemical characteristics of the stimulating compounds little more is known at present than in the case of the vertebrates. However, insects offer certain advantages in studies of this sort, such as the accessibility of the sensory organs, the possibility of avoiding the complications of salivary and nasal secretions and the availability of statistically useful numbers of animals, which invite more attention than the group has been accorded in the past. The fact that the basic features of gustation and olfaction in insects now appear to be not too different from those of the analogous processes in man should also encourage greater use of this advantageous material for fundamental studies of chemosensory function, the more so since the results may be expected to find immediate application in the fields of preventive medicine and agriculture through the development of improved insect attractants and repellents. Recent experiments on contact chemoreception in insects which bear on the problem of the relationship between chemical and physical properties, on the one hand, and stimulatory effect, on the other, are summarized below.

Acceptable compounds. Since von Frisch (63) reviewed the subject, work on the sugars has been amplified principally by the studies of Haslinger (76) with blowflies, Bauer (9) with water beetles, Schmidt (126) with ants and Thorpe *et al.* (136) with wireworms. It is now clear, as Weis (142) had already demonstrated for butterflies, that many insects accept a wider variety of sugars than bees, and that there is no hard and fast correlation between what is acceptable and what is nutritious. Equally clear, unfortunately, is the fact that no information has been obtained which would permit a revision of von Frisch's conclusion: that the factors involved in stimulation by sugars, in insects as in other animals, simply do not reach expression in our structural formulae.

Unacceptable compounds. Somewhat more encouraging progress has been made in the field of the unacceptable. For several series of salts with a common anion (acetates, bromides, chlorides, iodides, nitrates and sulfates), Frings (54) has shown that the efficiency in stimulation may be correlated directly with the mobilities of the cations, including H^+. These studies, conducted on the mouthparts of the American roach, were an outgrowth of earlier work on oral reception in *Cecropia* larvae (53), in which similar results were obtained, and have been extended by Frings and O'Neal (55) to the legs of the horsefly, *Tabanus sulcifrons*, and by Dethier (40) to the ovipositors of parasitic Hymenoptera. The relationship thus appears to be of rather general validity among insects and, according to Frings, is in substantial agreement with most of the data reported in the literature for chemical stimulation of other animals by the series of cations employed ($Li^+ < Na^+ < Mg^{++} < Ca^{++} = Sr^{++} < K^+ \leqq Cs^+ = Rb^+ < NH_4^+ <<< H^+$), including their gustatory effect in man. As pointed out by Frings, the order of effectiveness of the salts is also that of their partition coefficients, which parallels that of ionic mobilities (110). The exact modus operandi of stimulation by cations thus remains in doubt, since either penetration or adsorption at a lipoprotein interface would fit the facts (and as yet it is not known that either occurs); however, it is a step forward to have related their stimulatory effects quantitatively to some definite physical property. In regard to the anions the situation is more dubious. The work of Frings (54) establishes the following

empirical order of effectiveness: $PO_4^{---} < Ac^- < SO_4^{--} = Cl^- \leqq Br^- < I^- < NO_3^- << OH^-$, for which there is at present no obvious physical basis; it is to be hoped that further studies will shed light on this question, and on possible summation or antagonism between ions of the same and of opposite sign.

Progress has been reported also in work with organic compounds. Using flies which had been desensitized to odors by removal of the antennae and labella, Dethier and Chadwick (42) measured rejection thresholds for a series of aliphatic alcohols by presenting the compounds to the tarsi in 0.1 M sucrose. Exceptionally high correlations were found between the stimulating power of the compounds and such properties as boiling point, molecular area, oil-water distribution coefficients, molecular moments, vapor pressures and activity coefficients. Molecular weight, number of carbon atoms and osmotic pressure were eliminated from consideration in part by the use of isomers, which proved usually to have different thresholds, while the correlation with vapor pressures was inverse. It was concluded that surface energy relationships must be involved in stimulation. Similar studies now under way with aldehydes, ketones, glycols, bromo- and chloro-hydrins, cellosolves, carbitols, amines and amides have so far given concordant results, but the data are still incomplete. Tests with a number of fatty acids indicated that while the H ion is the principal factor in stimulation with compounds of this type, a contribution is made also by the anion(s) or undissociated acid and that this component is effective in inverse proportion to the hydrophile nature of the molecule (22). The relationship is thus very similar to that found with the same class of compounds for human taste (cf., e.g., Taylor *et al.*, 132) and for their penetration into other organisms (e.g., Crozier, 28).

Though all evidence at present available points to the importance of free energy relationships in the limiting process in contact chemical stimulation, a more exact definition of the mechanism is impeded by gaps in information relative to the physical characteristics of the compounds tested, by the lack of knowledge as to the nature of the receptor surface and by our complete ignorance of the essential features of the stimulatory event. Another obstacle is that the techniques at hand limit the investigations largely if not entirely to compounds soluble in water or in dilute sucrose, so that in most homologous series only a few members can be examined although it is recognized that many organic liquids, nearly insoluble in water, are highly stimulating when applied in the undiluted form. As indicated in a study of the glycols (43), in which it proved feasible to measure rejection thresholds for compounds with an average molecular weight as great as 1540, the possibility must be reckoned with that curves which appear linear when based on only the first few members of an homologous series may prove to have a different order of complexity when extended to include molecules of greater size.

At present, the one relationship which appears to reconcile all the known facts of contact chemoreception in insects, including those derived from studies of inorganic electrolytes, is between stimulating efficiency and lipid solubility or oil-water distribution coefficients. Unfortunately it is just in this area that reliable physical data are lacking for many of the compounds tested, and the existence of a lipoid layer or lipo-protein mosaic at the surface of or within the receptors is still wholly

hypothetical, plausible though it may be in view of recent work on the general surface of the insect cuticle (10, 147, 148). Exact studies of the constituents and structure of the receptor surface are urgently needed.

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USE OF MICROORGANISMS FOR ASSAY OF VITAMINS

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THE PRESENT WIDESPREAD USE of microorganisms for the determination of th water-soluble vitamins represents a practical application of an essentially academic study—the nutritive requirements of microorganisms. Of necessity, microbiologists were, until recent years, more interested in the development of practical media, concocted from crude materials, which would permit continued growth under laboratory conditions of the vast numbers of different organisms found in nature, than they were in defining the exact nutritive requirements of these cultures. Isolated and sporadic investigations of the latter subject were indeed made, especially with yeast, but serious investigation of the subject has been undertaken only within the past fifteen years. This period of intensive investigation thus coincided with that during which rapid advances in knowledge of animal nutrition were being made. As has been pointed out elsewhere (1–3) the resulting cross fertilization between the two fields has immeasurably speeded development of knowledge in both fields. It was found, in brief, that the vitamins required by animals and the growth factors required by certain microorganisms were, in many cases, identical. Demonstration of the essential nature of some of these substances for growth (e. g., thiamine, riboflavin, pyridoxine, choline) was made primarily with animals, of others (e.g., inositol, biotin, pantothenic acid, pyridoxal, pyridoxamine, p-aminobenzoic acid) primarily with microorganisms. Both animals and microorganisms are inextricably involved in the early history of nicotinic acid and folic acid.

For each of the B-vitamins known to be required for growth of animals, there are thus known several different microorganisms which also require that vitamin for growth. In many cases (e.g., biotin, 4; pantothenic acid, 5–7; folic acid, 8–11; inositol, 12), the growth responses of such organisms were adapted to provide a quantitative or semiquantitative measure of the amount of the growth factor present even before the responsible factor was identified, and this measure was used to guide the course of concentration from natural materials in the initial isolation of these substances. It is from such procedures, used to identify the growth factors essential for the various test organisms, that our present microbiological assay methods have developed.

In theory, any microorganism which, under a specified set of conditions, requires addition of a vitamin to the culture medium for growth, might be used for the estimation of that vitamin in natural materials. Examination of known nutritional requirements of microorganisms (reviews: 3, 13, 14, 15) reveal large numbers of different organisms which might be thus used for almost any of the known vitamins. In practice, however, comparatively few groups of organisms have been thus used. Various microbiological assay methods which have been proposed for individual vitamins are listed, together with incidental information about the methods, in tables 1, 2 and 3. It will be observed that the majority of methods utilize lactic acid bac-

TABLE 1. VITAMIN ASSAY METHODS USING LACTIC ACID BACTERIA

TEST ORGANISM	CONCENTRATION FOR STANDARD CURVE	INCUBATION TIME	MEASUREMENT OF RESPONSE	AUTHOR, DATE AND REFERENCE
A. Biotin				
	γ per 10 cc.	*hrs.*		
Lactobacillus arabinosus	0–0.001	72	acidimetric	Snell and Wright, 1941 (16)
Lactobacillus arabinosus	0–0.002	72	acidimetric	Wright and Skeggs, 1944, (17)
Lactobacillus casei	0–0.002	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.001	72	acidimetric	Shull *et al.*, 1942–3 (19, 20)
Lactobacillus casei	0–0.0012	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
Streptococcus faecalis	0–0.0015	16–20	turbidimetric	Luckey *et al.*, 1946 (22)
Streptococcus faecalis	0–0.001	20	turbidimetric	Rabinowitz and Snell, 1947 (23)
B. Folic acid (pteroylglutamic acid)				
Lactobacillus casei		72	acidimetric	Snell and Peterson, 1940 (24)
Lactobacillus casei	0–0.0015	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.0015	72	acidimetric	Bird *et al.*, 1945 (25)
Lactobacillus casei	0–0.001	30–72	acidimetric	Teply and Elvehjem, 1945 (26)
Lactobacillus casei	0–0.0012	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
Streptococcus faecalis	0–0.0015	16	turbidimetric	Mitchell and Snell, 1941 (27)
Streptococcus faecalis	0–0.002	16	turbidimetric	Luckey *et al.*, 1944 (28)
Streptococcus faecalis	0–0.008	30–72	acidimetric	Teply and Elvehjem, 1947 (26)
Streptococcus faecalis	0–0.008	20	turbidimetric	Rabinowitz and Snell, 1947 (23)
C. Nicotinic acid				
Lactobacillus arabinosus	0–0.5	72	acidimetric	Snell and Wright, 1941 (16)
Lactobacillus arabinosus	0–0.5	72	acidimetric	Krehl *et al.*, 1943 (29)
Lactobacillus arabinosus	0–0.5	72	acidimetric	Barton-Wright, 1944 (30)
Lactobacillus arabinosus	0–0.5	40–72	acidimetric	Sarett *et al.*, 1945 (31)
Lactobacillus arabinosus	0–0.5	72	acidimetric	(Collaborative, 1945 (32) (USP-AOAC))
Lactobacillus arabinosus	0–0.5	72	acidimetric	Lawrence *et al.* 1946 (33)
Lactobacillus casei	0–0.8	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.2	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
Leuconostoc mesenteroides	0–2.0	72	acidimetric	Johnson, 1945 (34)
Streptococcus faecalis	0–0.5	20	turbidimetric	Rabinowitz and Snell, 1947 (23)
D. Pantothenic acid				

Leuconostoc mesenteroides				Rabinowitz and Snell, 1947 (23)
Streptococcus faecalis	0–0.5	20	turbidimetric	

D. Pantothenic acid

Lactobacillus casei	0–0.2	72	acidimetric	Snell *et al.*, 1937 (6, 35)
Lactobacillus casei	0–0.2	72	turbidimetric or acidimetric	Pennington, *et al.*, 1940 (36)
Lactobacillus casei	0–0.15	72	acidimetric	Strong *et al.*, 1941 (37)
Lactobacillus casei	0–0.20	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.10	72	acidimetric	Neal and Strong, 1943 (38)
Lactobacillus casei	0–0.10	72	acidimetric	Ives *et al.*, 1945 (39)
Lactobacillus casei	0–0.20	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
Lactobacillus arabinosus	0–0.2	72	acidimetric	Snell and Wright, 1941 (16)
Lactobacillus arabinosus	0–0.2	18–72	turbidimetric or acidimetric	Skeggs and Wright, 1944 (40)
Lactobacillus arabinosus	0–0.2	14–72	turbidimetric or acidimetric	Hoag *et al.*, 1945 (41)
Streptococcus faecalis	0–0.4	20	turbidimetric	Rabinowitz and Snell, 1947 (23)

E. Riboflavin

Lactobacillus casei	0–0.3	72	acidimetric	Snell and Strong, 1939 (42)
Lactobacillus casei	0–0.4	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.15	72	acidimetric	Greene and Black, 1943 (43)
Lactobacillus casei		72	acidimetric	Barton-Wright and Boothe, 1943 (44)
Lactobacillus casei	0–0.2	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)

F. Thiamine

Streptococcus salivarius	0–0.002	24	turbidimetric or acidimetric	Niven and Smiley, 1943 (45)
Lactobacillus fermenti	0–0.050	16–18	turbidimetric	Sarett and Cheldelin, 1944 (46, 47)

G. Vitamin B_6

1. Pyridoxal

Lactobacillus casei	0–0.10	24–36	turbidimetric	Snell and Rannefeld, 1945 (48)
Lactobacillus casei	0–0.04	24	turbidimetric	Rabinowitz *et al.*, 1947 (49)

2. Pyridoxal plus pyridoxamine

Streptococcus faecalis	0–0.004	16	turbidimetric	Snell and Rannefeld, 1945 (48)
Streptococcus faecalis	0–0.006	22–24	turbidimetric	Rabinowitz and Snell, 1947 (23)

H. p-Aminobenzoic acid

Lactobacillus arabinosus	0–0.0005	72	acidimetric	Lewis, 1942 (50)
Leuconostoc mesenteroides	0–0.001	15	turbidimetric	Pennington, 1946 (51)

TABLE 2. VITAMIN ASSAY METHODS USING YEASTS

TEST ORGANISM	CONCENTRATIONS FOR STANDARD CURVE	INCUBATION TIME	MEASUREMENT OF RESPONSE	AUTHOR, DATE AND REFERENCE
A. Biotin				
	γ per 10 cc.	*hrs.*		
Saccaromyces cerevisiae	0–0.0002	16	turbidimetric	Snell *et al.*, 1940 (52)
Saccaromyces cerevisiae	0–0.0004	18–22	turbidimetric	Hertz, 1943 (53)
Saccaromyces cerevisiae	0–0.001	16–18	turbidimetric	Atkin *et al.*, 1944 (54)
B. Nicotinic acid				
Zygosaccharomyces marxianus	0–0.2	72	turbidimetric	Leonian and Lilly, 1945 (56)
Torula cremoris	0–0.2	12–40	turbidimetric	Williams, 1946, (57)
C. Pantothenic acid				
Saccharomyces carlsbergensis	0–0.4	16–18	turbidimetric	Atkin *et al.*, 1944 (58)
D. Thiamine				
Saccharomyces cerevisiae	0–0.01	15	turbidimetric	Williams *et al.*, 1941 (59)
Saccharomyces cerevisiae	0.5–2.0	3	CO_2 evolution	Schultz *et al.*, 1942 (60)
Saccharomyces cerevisiae	0.01–0.02	3	CO_2 evolution	Atkin *et al.*, 1939 (61); Josephson and Harris, 1942 (62)
Endomyces vernalis	0–0.1	24	turbidimetric	Woolley, 1941 (63, 64)
E. Vitamin B_6 (pyridoxal plus pyridoxamine plus pyridoxine)				
Saccharomyces cerevisiae	0–0.002	15	turbidimetric	Williams *et al.*, 1941 (65)
Saccharomyces cerevisiae	0–0.004	16–18	turbidimetric	Siegel *et al.*, 1943 (66)
Saccharomyces carlsbergensis	0–0.040	16–18	turbidimetric	Atkin, *et al.*, 1943 (67) (55)
Saccharomyces oviformis	0–0.008	20	turbidimetric	Burkholder, 1943 (68)
F. Inositol				
Saccharomyces cerevisiae	0–20	16	turbidimetric	Woolley, 1941 (69)
Saccharomyces cerevisiae	0–5	15	turbidimetric	Williams *et al.*, 1941 (70)
Saccharomyces carlsbergensis	0–10	16–18	turbidimetric	Atkin *et al.*, 1944 (55)
G. General method for all six of the above vitamins				
Kloeckera brevis			turbidimetric	Burkholder *et al.*, 1944 (71)

teria as test organisms. Yeasts and *Neurospora* mutants are also widely used. The extensive use of lactic acid bacteria for such assays is due in part to the historical accident that knowledge of their nutritional requirements was sufficiently advanced to permit application to bioassay at a time when need for such procedures became most acute. Consequently, they were among the first organisms to be thus applied. The specific and complex nature of their nutritional requirements, their rugged nature, and the fact that their growth could be easily followed either by turbidimetric means or by titration of the lactic acid produced during growth, have served to keep this group of organisms foremost among those used for assay both of vitamins (3, 13) and of amino acids (reviews: 96, 97, 98). Yeasts and *Neurospora* mutants have provided not only excellent alternative methods for some of the vitamins required by lactic acid bacteria but are applicable to the determination of certain vitamins not required for growth of the latter organisms.

In addition to the original descriptions of the various proposed microbiological methods referred to in tables 1 to 3, many of these procedures have been critically discussed and described in detail or in summary form in recent monographic treatments (99–101). Except for illustrative purposes, the present review will avoid these technical details and will attempt, rather, to present the basic information necessary to the development, understanding and evaluation of various assay procedures. Before this can be done effectively, it will be necessary to have in mind the general procedure used in carrying through a microbiological assay.

In general, the procedure used in each of the assay methods is similar. The essential steps, common to all methods, but modified in detail according to the test organism being used and the vitamin to be determined, can best be illustrated by a brief description of an actual example. For this purpose, the microbiological method of Snell and Strong (42) for riboflavin will serve. This procedure was the first microbiological method for the routine determination of a vitamin in natural materials to achieve widespread use and general acceptance. The use of lactic acid bacteria for such assays, and many of the general techniques so widely followed today, were introduced with this method, itself an adaptation of earlier procedures used in studying growth factor requirements of these organisms (3, 6, 95). The history, procedural details, range of applicability and evidence for the specificity and validity of this method have been considered in detail elsewhere (100–102).

The test organism, *Lactobacillus casei*, is carried as stab cultures by monthly transfer in a yeast dextrose agar. Such cultures are incubated at 37°C. for 24 to 48 hours, or until good growth along the line of the stab is visible, and are then held in the refrigerator for the remainder of the interval between transfers. About 24 hours before an assay is to be made, a transfer is made from this culture to a tube of sterile inoculum medium. This consists of the riboflavin-deficient basal medium supplemented with riboflavin (1 γ per 10 cc.). This inoculum culture is then incubated at 37° C. until used.

The assay is conveniently carried out in 16 x 180 mm. lipless test tubes, held in one or more metal racks which are easily autoclaved. To one series of tubes, increasing amounts of a standard solution of riboflavin (from 0 to 0.25 γ in increments of about 0.05 γ) are added. To other similar series, increasing amounts of extracts

Kloeckera brevis

TABLE 3. VITAMIN ASSAY METHODS USING MOLDS AND MISCELLANEOUS MICROORGANISMS

TEST ORGANISM	CONCENTRATION FOR STANDARD CURVE	INCUBATION TIME	MEASUREMENT OF RESPONSE	AUTHOR, DATE AND REFERENCE
A. Biotin				
	γ per 10 cc.	*hrs.*		
Neurospora crassa	0–0.002	72	weigh mycelium	Hodson, 1945 (72)
Neurospora crassa	0–0.002	72	weigh mycelium	Tatum *et al.*, 1946 (73)
Clostridium butylicum	0–0.01	72	turbidimetric	Lampen *et al.*, 1942 (74)
Rhizobium trifolii	0–0.003	120	turbidimetric	West and Woglom, 1942 (75)
B. Nicotinic acid				
Proteus HX 19	0–0.12	36	turbidimetric	Lwoff and Querido, 1939 (76); Morel, 1943 (77)
Proteus HX 19	0–0.10	40	turbidimetric	Grossowicz and Sherstinsky, 1947 (78)
Shigella paradysenteriae	0–0.25	16–22	turbidimetric	Isbell *et al.*, 1941 (79)
Shigella paradysenteriae	0–0.01	96	acidimetric	Dorfman *et al.*, 1940 (80, 81)
C. Pantothenic acid				
Proteus morganii	0–0.005	24	turbidimetric	Pelczar and Porter, 1941 (82)
D. Thiamine				
Phycomyces blakesleeanus	0–0.2	144–480	weigh mycelium	Schopfer, 1935–1945 (83, 84)
Phycomyces blakesleeanus	0–0.2	336	weigh mycelium	Hammer *et al.*, 1943 (85)
Staphylococcus aureus	0–0.005	36	turbidimetric	West and Wilson, 1938 (86)
E. Vitamin B_6 (pyridoxal plus pyridoxamine plus pyridoxine)				
Neurospora sitophila (mutant)	0–1.0	120	weigh mycelium	Stokes *et al.*, 1943 (87)
Neurospora sitophila (mutant)	0–0.07	72	weigh mycelium	Tatum *et al.*, 1946 (73)

F. Aminobenzoic acid				
Actobacter suboxydans	0–0.01	48	turbidimetric	Landy *et al.*, 1942–3 (88, 89)
Acetobacter suboxydans	0–0.01	60–70	turbidimetric	Cheldelin and Bennett, 1945 (90)
Clostridium acetobutylicum	0–0.002	20–24	turbidimetric	Lampen and Peterson, 1944 (91)
Neurospora crassa (mutant)	0–0.03	20	diameter of mycelial growth	Thompson *et al.*, 1943 (92)
Neurospora crassa (mutant)	0–0.05	72	weigh mycelium	Tatum *et al.*, 1946 (73)
G. Choline				
Neurospora crassa (mutant)	0–8	72	weigh mycelium	Horowitz and Beadle, 1943 (93)
Neurospora crassa (mutant)	0–8	120	weigh mycelium	Hodson, 1945 (72)
Neurospora crassa (mutant)	0–10	72	weigh mycelium	Tatum *et al.*, 1946 (73)
I. Inositol				
Neurospora crassa (mutant)	0–15	72	weigh mycelium	Beadle, 1942 (94)
Neurospora crassa (mutant)	0–10	72	weigh mycelium	Tatum *et al.*, 1946 (73)
J. General method for p-aminobenzoic acid, biotin, vitamin B_6, choline and inositol				
Neurospora (mutants)		72	weigh mycelium	Tatum *et al.*, 1946 (73)

of the various samples are added in volumes (<5.0 cc.) considered by approximation to furnish amounts of the vitamin between 0.05 and 0.25 γ. All tubes are diluted with distilled water to 5 cc., then 5 cc. of the double strength, riboflavin-deficient medium are added. The tubes are plugged with cotton or otherwise appropriately covered and sterilized by autoclaving at 15 pounds pressure for 15 minutes. After cooling to body heat or below, each tube is inoculated with one drop of the turbid inoculum culture. The racks are then incubated at 37° C. The response to riboflavin may be measured turbidimetrically after 24 to 30 hours incubation, but is more commonly determined by titrating the lactic acid produced after 72 hours incubation.

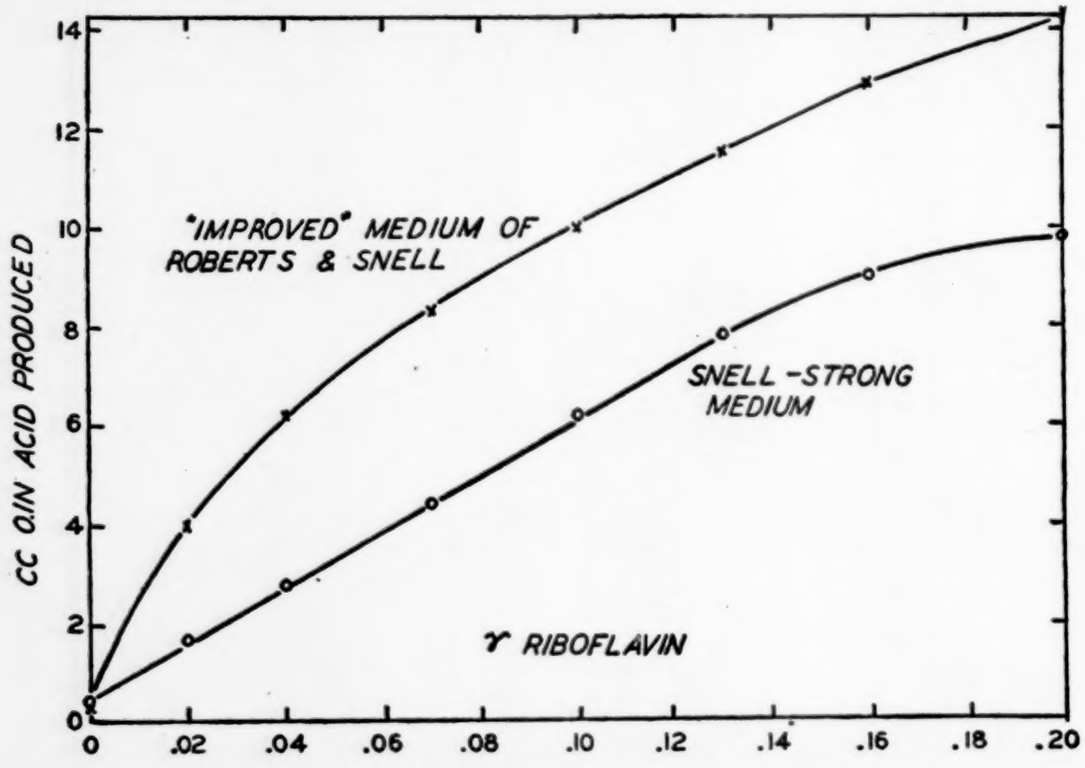

FIG 1. RESPONSE OF LACTOBACILLUS CASEI to riboflavin in two different media

To obtain the assay results, a standard curve is commonly plotted relating the response of the test organism (in turbidity units or cubic centimeters of alkali used in titration) to the concentration of riboflavin. The lower curve of figure 1 is an example of such a standard curve. The riboflavin content of the various aliquots of sample used may be obtained by interpolating the response obtained in tubes containing these aliquots onto the standard curve. Results falling outside the assay range (0.05–0.25 γ of riboflavin per tube) are discarded. At least three different sized aliquots of the sample should contain amounts of riboflavin within this range. The riboflavin content of the sample is then calculated from that of each aliquot. The figures obtained should agree within ±10 per cent and should show little or no tendency to increase or decrease regularly with increasing size of the aliquot ('drift'—see further discussion below). If the values meet these conditions, they are averaged to obtain the final result.

From this example we may now outline the essential steps involved in this or any

similar assay. These are: *a*) preparation of media for carrying stock cultures and maintenance of these cultures; *b*) preparation of the vitamin-deficient basal medium; *c*) preparation of inoculum medium and of the inoculum culture; *d*) extraction of the vitamin from the sample preparatory to assay; *e*) setting up the assay; *f*) sterilization of the assay tubes and media; *g*) inoculation with the test organism; *h*) incubation; *i*) determination of response to the vitamin and the vitamin-containing extracts; and *j*) calculation of the results.

The complexity of this series of steps is such that the end result may be influenced by the manner in which almost every operation of the procedure is carried out. In many (perhaps most) cases, the effect of variation in procedure at any of the various steps has not been studied. Conditions have been selected which appear optimum and which give consistent results. The fundamental assumption is made that any uncontrolled variations in procedural details which occur under conditions of normal practice will affect the response of the test organism alike to standard and sample, and hence will not materially affect the accuracy of the assay result. Certainly, in most cases, this assumption appears to be borne out in practice. It will, however, be useful to point out instances in which variation at the various stages of an assay are known to affect the results obtained.

PHYSICOCHEMICAL REQUIREMENTS FOR GROWTH OF THE VARIOUS ASSAY ORGANISMS. Steps (*a*) through (*c*) of the above generalized procedure involve knowledge of the nutritional requirements and general cultural conditions necessary for growth of the various test organisms. Of the three widely used groups, *Neurospora* has the simplest nutritional requirements. Butler, Robbins and Dodge (104) and Beadle and Tatum (105) showed that wild type strains of this organism required biotin as the sole growth factor of vitamin-like nature, and that if this were supplied, these organisms grew well on a medium containing a carbon and energy source (e.g., sucrose), an inorganic source of nitrogen (ammonium salts) and several inorganic salts. Such wild types could, of course, be used only for assay of biotin. The present use of these organisms for assay stems from the work of Beadle and Tatum (105) who showed that, by irradiation with X-rays or ultraviolet light, mutant strains of the organism could be derived which were unable to synthesize other vitamins or amino acids and therefore required addition of these substances to the minimal basal medium for growth. In a recent tabulation, Beadle and Tatum (106) list individual mutants which require, in addition to biotin, one of the following vitamins: thiamine, riboflavin, vitamin B_6, nicotinic acid, pantothenic acid, p-aminobenzoic acid, inositol and choline. Thus all of the presently characterized B-vitamins, folic acid excepted, could be determined with these organisms, if desired. As is apparent from table 3, several such applications have been made.

Somewhat more complex nutritionally are the various yeasts. The nutritional requirements of these organisms have been extensively investigated. Inositol (12), thiamine (107), biotin (4), pantothenic acid (5, 108) or its cleavage product, β-alanine (109), vitamin B_6 (110, 111, 48) and nicotinic acid (71, 112, 113) have been identified as necessary or stimulatory for various yeasts. Different species or strains of yeast require different combinations of these six vitamins for growth. As an example, the work of Burkholder *et al.* (71) may be cited. Of 163 strains of yeasts belonging in

110 named species and varieties, 86 required one or more of the six vitamins mentioned above, as follows: biotin, 78; thiamine or its split products, 33; pantothenic acid (or β-alanine), 30; inositol, 15; nicotinic acid, 13; vitamin B_6, 13. Some species required no vitamins at all (e.g., *Hansenula lambica*); others required all of the six vitamins (e.g., *Kloeckera brevis*. Between these two extremes, other species required from one to five vitamins in 18 different combinations. Only a single yeast, *Rhodotorula aurantiaca*, has been found which requires p-aminobenzoic acid for growth (114), and none are yet known which require riboflavin or folic acid.

In the presence of a complete assortment of vitamins, yeast, like *Neurospora*, will grow when furnished with the appropriate inorganic salts and a fermentable carbohydrate (e.g., glucose or sucrose). Ammonium salts are a commonly used nitrogen source. Although good growth can be obtained on such minimal media, and no yeasts have been reported to require preformed amino acids, it is found quite generally that addition of amino acids often improves growth considerably (71, 53, 115). For this reason, protein hydrolysates are often added to assay media employing yeasts as the test organisms.

By far the most complex nutritionally of the microorganisms used for bioassay are the lactic acid bacteria. The nutritional requirements of those members of the group which are used for assay are fairly well known and have been summarized elsewhere (3, 13, 96, 101). Vitamins required for growth include biotin, folic acid, nicotinic acid, pantothenic acid, riboflavin, thiamine, vitamin B_6 and p-aminobenzoic acid. No known culture requires all of these. Those with simpler requirements, such as *L. arabinosus*, require only biotin, nicotinic acid and pantothenic acid; others with more complex requirements (e.g., *L. casei*) may require in addition folic acid, riboflavin and vitamin B_6. Other combinations of requirements also exist. No organisms of this group are known which require inositol or choline for growth.

In addition to various vitamins, these organisms require a variable assortment of amino acids, certain purine and pyrimidine bases, a fermentable carbohydrate and certain mineral salts for growth. Under defined conditions, many cultures are stimulated by substances which are still unidentified.

To obtain good growth of any of these organisms, it is necessary to satisfy other physiological requirements. Thus, yeasts and *Neurospora* grow best under aerobic conditions. Stock cultures of these organisms are therefore carried on agar slants, exposed to air. In assay work, similarly, these organisms are cultured in vessels where the ratio of surface to volume of the culture medium is high, to permit ready access of air. Alternatively, with yeasts, where the procedure does not interfere with subsequent determination of growth, aeration may be effected by active shaking during the course of incubation.

Lactic acid bacteria, on the other hand, grow essentially anaerobically, although small amounts of air are not toxic, that is, these organisms are microaerophilic or facultatively aerobic (103). Aeration by shaking or exposure of large surfaces to air is generally deleterious. For this reason, stock cultures are carried as stabs in agar, and for assay purposes, ordinary test tubes serve well as containers since the organisms grow well throughout a deep tube of medium. These organisms produce large amounts of lactic acid during growth, and to prevent the pH of the medium from

rapidly dropping to levels which would inhibit growth (varying with different organisms from pH 3.0 to about 4.3 (116)), buffers must be added to the assay medium. Some of the organisms (e.g., *Streptococcus lactis*, *Leuconostoc mesenteroides*) are inhibited by much smaller hydrogen ion concentrations than are others (e.g., *Lactobacillus arabinosus*, *L. casei*, *L. delbrueckii*), so that to achieve high acid production by the former group it is necessary to pay careful attention to the type and amount of buffer present. In a nutritionally adequate medium, the amount of acid produced by a given organism can often be increased by increasing the amount of sugar, or of buffer, or both.

Other important factors to be controlled in use of organisms for quantitative assay work are the temperature of growth and the initial pH of the medium. It has been general practice to incubate assays at the assumed 'optimum temperature' of the test organism, although this temperature has never been accurately determined under assay conditions, and may well vary with the same organism when used in different basal media with different limiting nutrients. Such comparisons as have been made (e.g., 117, 118) indicate that the exact temperature selected for incubation is not of crucial importance, so long as it lies reasonably near to the optimum temperature, in a range which permits good growth of the test organism. Since the rate of growth of an organism is a function of temperature, it is important that the temperature selected for incubation be maintained constant throughout the area occupied by the assay vessels. Temperatures most extensively used with various lactic acid bacteria are from 30 to 37°C., varying somewhat with the organisms. Yeasts and *Neurospora* are most commonly incubated at 30°C., although lower temperatures have sometimes been used, especially with the former organisms.

The optimum pH for growth of most lactic acid bacteria is stated to lie between 5.5 and 6.5 (119, 120). Since some acid production occurs during autoclaving, the initial pH of the medium should be somewhat higher than this, 6.8 to 7.0 (119), and this is common practice. Like other fungi, yeasts and *Neurospora* grow over a wide range of hydrogen ion concentrations. Ordinarily, the initial pH of the media used with these organisms is about 5.0.

With this basic information about the nutritive requirements of cultures in mind, it is possible to proceed to consideration of the individual steps involved in microbiological assay, enumerated earlier.

A. *Carrying stock cultures of test organisms.* In all cases, it is essential that pure cultures of the test organism be maintained. If, after a period during which successful assays have been obtained, sudden transition to irregular behavior is noted, the purity of the stock culture may be suspected. For carrying cultures, the customary media and procedures long followed by bacteriologists suffice. Although, in tracing the history of the use of an individual organism for assay, it will often be found that modifications in the composition of the medium for carrying stock cultures have been introduced, these have usually been made arbitrarily, on *a priori* grounds, and have not been shown to result in improved assays. In a recent study (121), however, it was found that the magnitude of response of two commonly used assay organisms, *Lactobacillus arabinosus* and *Lactobacillus casei*, to limiting amounts of nicotinic acid or of riboflavin, decreased after long periods of time if these organisms were trans-

ferred monthly in a yeast extract-dextrose-agar, as is common practice. Such cultures could be 'rejuvenated' by a series of transplants in an enriched medium containing peptones and a liver extract.

In an independent investigation (46) dealing with thiamine assay with *Lactobacillus fermenti*, the customary yeast-glucose-agar was recommended for carrying this culture. In subsequent assays conducted much later, it was noted (47) that excessive growth occurred in assay tubes containing no added thiamine. This was attributed to development of a limited capacity of the test organism to synthesize thiamine when carried in a thiamine-low medium. This tendency was eliminated by enriching the medium used with an excess of thiamine (47).

Such experiments illustrate that the medium in which stock cultures are carried may affect assays subsequently carried out with the assay organisms, and point to the possibility of improving some methods by systematic investigation of this neglected aspect of the overall procedure. It might be similarly expected that frequency of transfer, incubation temperatures, etc., might similarly affect subsequent growth behavior. Again, these factors have not been investigated.

Recently, it was found possible to use lyophilized cultures of microorganisms as inoculum for certain assays (121). If generally successful, such procedures would relieve laboratories conducting occasional assays of the burden of carrying stock cultures and would make possible use of an inoculum of constant physiological activity from place to place and from time to time.

B. *Preparation of the vitamin-deficient basal medium.* The nature of the vitamin-free basal medium is certainly one of the most important single factors which determine the accuracy of an assay method. It is not surprising, therefore, that successive modifications of a method, which appear after its introduction, are most frequently concerned with the nature of the basal medium. What, then, are the essential characteristics of a satisfactory basal medium? It must, of course, be free of the substance to be determined. It must also be sufficiently complete, in a nutritional sense, so that addition of the sample, in the amounts necessary for assay, will not supply substances other than the essential vitamin which increase or decrease the amount of growth (or other measured quantity) obtained at the end of the recommended incubation period. Finally, the amount of growth (or other product) produced by additions of graded amounts of the vitamin must be sufficient for accurate measurement by the method adopted.

The adequacy of a basal medium for assay thus cannot be judged apart from other experimental details. A basal medium, which, when supplemented with the missing essential nutrient, contains all of the substances *essential* for growth, but is lacking in one or more substances which markedly stimulate rate of growth, may give very satisfactory assays if the period of incubation is sufficiently long to eliminate the effect of nonessential growth stimulants, so that the essential substance being assayed is the only substance limiting growth. Such a medium will also be satisfactory for assaying samples which are rich in the vitamin to be determined and hence are added in amounts too small to supply interfering materials. Satisfactory assays can also be obtained by removing the interfering materials from the extract being assayed. Conversely, such a medium will tend to give erroneous results if used with a short

incubation period, and with samples low in the substance being determined or high in other growth stimulants not present in the medium. Where possible, of course, the most satisfactory way to eliminate the effects of growth stimulants present in samples for assay is to add these to the basal medium, either as pure compounds or as crude extracts suitably freed from the vitamin to be determined.

Examples in support of the above general statements may now be cited. In assay of vitamin B_6 in yeast extract with *Streptococcus faecalis*, Rabinowitz and Snell (23) noted high results following incubation periods of 12 to 20 hours. If the incubation period was extended to 22 hours or longer, constant assay values of the proper magnitude were obtained. The effect of stimulating substances present in the extract but not in the medium, and which contributed to the value found for vitamin B_6 after short incubation periods, was eliminated by extending the incubation period. It is well known that fatty materials markedly interfere with the determination of riboflavin and pantothenic acid with lactic acid bacteria (122–125). These effects are prevented by removing them from the extracts, either by filtration at pH 4.5, by extraction with ether, or both (122, 125). Skeggs and Wright (40) have shown, however, that it is also possible to avoid these effects (in pantothenic acid assay with *Lactobacillus arabinosus*) by adding oleic acid to the assay medium. An additional instance of the same nature is afforded by observations of Stokes *et al.* (87) that the presence of thiamine in extracts stimulated response of *Neurospora sitophila* 'pyridoxineless' to vitamin B_6, with consequent high results. These authors avoided this source of error by destroying the thiamine in each sample by sulfite treatment, a procedure which left the vitamin B_6 undamaged. Tatum *et al.* (73) showed that interference from this source could also be eliminated by the simple expedient of adding an excess of thiamine to the basal medium.

In attempting to improve previously devised basal media, it has often been assumed by investigators that any modification which will permit increased growth (or acid production, etc.) of the test organism in the presence of an excess of the vitamin to be determined will increase the specificity or accuracy of an assay method; mere obtention of such increased growth has been deemed sufficient to prove the modified medium to be an 'improved' medium for assay. Although, in some cases, the assumption appears logical, in no case have the necessary data to prove it been published. In an investigation bearing on this point, Roberts and Snell (21) devised a medium which permitted more rapid growth and higher acid production by *Lactobacillus casei* in response to added riboflavin than did the widely used medium of Snell and Strong (42). The dose response curves obtained in parallel trials on the two media are shown in figure 1. Despite the more luxuriant growth obtained in the modified medium, a series of parallel assays on the two media gave identical results, within experimental error of either method. The modified medium did permit assays to be obtained in less time, however. A similar result was obtained by Sarett *et al.* (31) in comparative assays for nicotinic acid with three different assay media. In a third instance, it was found (53) that supplementation of a previously devised assay medium for biotin (52) with hydrolyzed casein considerably increased the range of concentration over which growth of yeast depended on the biotin concentration. In the modified medium, the amount of growth obtained and the assay

range for biotin were both increased; the modification was therefore valuable since it increased the convenience of use. The conclusion (53) that the modified method has increased specificity over the original method due to elimination of nonspecific stimulation which might result from the amino acid content of samples would appear invalid, however, in view of comparative curves presented with the modified method, which showed that addition of excess hydrolyzed casein did not influence the response to biotin over the assay range used in the original method.

Once a medium and technique have been devised which afford assays of proven accuracy over a convenient concentration range with gradations of growth in an easily measurable range, it would appear that other data besides a demonstrated superiority in the maximum levels of growth (or acid production, etc.) permitted by the medium should be required to prove the superiority of a modified procedure. This is not to deny that an adequate assay medium should promote excellent growth when supplemented with the missing vitamin, but merely to emphasize that the prime objective is not obtention of maximum growth, but rather of accurate and convenient assays, and that the two goals are not *necessarily* identical.

Finally, it should be re-emphasized that in general, the complexity of basal media required by various organisms reflects the complexity of their nutritional requirements. This is well illustrated by the composition of four assay media for biotin, given in table 4; that used with *Lactobacillus arabinosus* is most complex, those used with yeast occupy an intermediate position and that used with *Neurospora* is simplest. Simplicity in minimal nutritive requirement is no assurance, however, that simple media will suffice for accurate assay of an essential nutrient in complex natural mixtures. Thus, Thompson *et al.* (92) found a fairly complex medium necessary to permit accurate assay for p-aminobenzoic acid with *Neurospora crassa* p-aminobenzoicless, and simple minimal media have not proved generally adequate for assay of various amino acids required by certain *Neurospora* mutants (126). Similarly, the early yeast growth method for pantothenic acid (5), by means of which pantothenic acid was originally discovered, and which served admirably as a guide for purification and near isolation of this substance, is completely unsatisfactory for its routine determination in natural materials (36) because of the presence in these materials of substances other than pantothenic acid which affect growth of yeast in the minimal media originally used. Very satisfactory, though more complex, media for determination of pantothenic acid in natural materials with yeast have since been developed (58).

C. *Preparation of the inoculum medium and inoculum culture.* With lactic acid bacteria, which are carried as stab cultures, it is necessary to grow an inoculum culture in liquid medium for use in inoculating the assay tubes. Most commonly, the vitamin-deficient basal medium, supplemented with adequate amounts of the missing vitamin to support growth, is used for this purpose, although sometimes a special medium is used. Almost no information is available concerning the effect of variation in composition of this medium on success of the subsequent assay. Nebulous evidence suggests, however, that growing the inoculum in an 'enriched' inoculum medium may produce improved growth in the subsequent assay, due presumably to carry over of stimulating factors not supplied by the basal medium. In some cases

(particularly pantothenic acid, biotin and folic acid), the cells may store sufficient amounts of the vitamin so that subsequent use of heavy inocula results in considerable growth in unsupplemented assay tubes. This can be prevented by using a

TABLE 4. COMPARATIVE ASSAY MEDIA USED FOR DETERMINATION OF BIOTIN WITH DIFFERENT TEST ORGANISMS

CONSTITUENT	*Lactobacillus arabinosus* (17)	*Saccharomyces cerevisiae* (53)	*Saccharomyces cerevisiae* (52)	*Neurospora crassa* (73)
	Amount per 100 cc. of double strength medium			
Glucose	4.0 gm.			
Sucrose		3.4 gm.	3.4 gm.	4.0 gm.
Acid-hydrolyzed casein (charcoal-treated)	1.0 gm.	0.1 gm.		
Ammonium sulfate		0.5 gm.	0.5 gm.	
Ammonium tartrate				1.0 gm.
Sodium acetate	1.2 gm.			
Aspartic acid		17 mg.	17 mg.	
Cystine and tryptophane	20 mg. ea.			
Adenine, guanine, xanthine, uracil	1.0 mg. ea.			
p-Aminobenzoic acid	20 μg.			
Calcium pantothenate	200 μg.	50 μg.		
β-Alanine			50 μg.	
Inositol		900 μg.	900 μg.	
Pyridoxine	400 μg.	100 μg.	3 μg.	
Nicotinic acid, riboflavin	200 μg.			
Thiamine	200 μg.	100 μg.	3 μg.	
KH_2PO_4	100 mg.	340 mg.	340 mg.	200 mg.
K_2HPO_4	100 mg.			
$MgSO_4 \cdot 7H_2O$	40 mg.	45 mg.	45 mg.	100 mg.
$CaCl_2 \cdot 2H_2O$		45 mg.	45 mg.	20 mg.
NH_4NO_3				200 mg.
Trace elements	[1]	[2]	[2]	[3]

[1] $MnSO_4 \cdot 4H_2O$, NaCl, $FeSO_4 \cdot 7H_2O$, 2.0 mg. each.
[2] $MnCl_2$, H_3BO_3, $ZnSO_4$, $TlCl_3$, 180 μg. each; $FeCl_3$, 90 μg; $CuSO_4 \cdot 5H_2O$, KI, 18 μg. each.
[3] NaCl, 20 mg.; B, 2 μg.; Mo, Mn, 4 μg. each; Fe, 40 μg.; Cu, 20 μg.; Zn, 400 μg.

diluted, washed inoculum (e.g., 21, 40) or by growing the inoculum in a medium containing barely enough of the vitamin to be assayed to permit good growth. In other assays (e.g., riboflavin, nicotinic acid), large inocula may be used without danger of 'carry over.' It is generally agreed that least 'lag' in growth of subsequent assays

occurs if inoculation is made from inoculum cultures which are in the actively growing, logarithmic phase (cf. 41).

With yeasts and *Neurospora* cultures, inocula are usually prepared directly from stock cultures by removing a portion of growth (cells or spores, respectively) from a slant and suspending in water. With the former organisms, it is now most common to use freshly prepared (i.e., 24 to 36 hours old) slant cultures for this purpose (e.g., 54, 58, 65), although older cultures will also serve (52). Further work on this phase of microbiological assay, to establish the preferable procedures, would likely be worthwhile.

D. *Extraction of the vitamin from the sample preparatory to assay.* It is now well known that the majority of the water-soluble vitamins occur in tissues in combined or 'bound' forms, which are often unavailable to microorganisms used for assay. An essential phase of any microbiological assay method, therefore, is development of a procedure for liberating the free vitamin from such combined forms. Solution of this problem is also necessary, of course, for valid application of chemical procedures for the estimation of vitamins. Properly speaking, it should be considered as an independent problem, apart from the method subsequently used to estimate the vitamin; and in comparing the merits of two assay media, the same extraction procedures should be used for preparation of samples in both cases. It is apparent that, if inadequate extraction procedures are employed, no assay medium, however good, can yield reliable values.

The object, then, of a good extraction procedure, is obtention of all of the vitamin of a sample in a form utilizable by the test organism, with the elimination so far as possible, of known interfering materials. The state of combination of a given vitamin, however, apparently is not the same in different tissues. Pantothenic acid, for example, is fully extracted from wheat in a form utilizable by yeast by water alone, whereas a preliminary enzymatic digestion is required to permit its complete extraction from corn (58). Similarly, different assay organisms differ in their ability to utilize naturally occurring, combined forms of a vitamin. Biotin in some yeast extracts, for example, is fully utilized by *Lactobacillus casei*, but the same extract requires hydrolysis with strong acid to permit full utilization of its biotin by *Lactobacillus arabinosus* (17). Many other similar examples might be cited. Thus an extraction procedure which is adequate for one set of samples and one test organism may be quite inadequate with different samples or with a different test organism. In the absence of special information, one should apply the most thoroughly tested and widely applicable extraction procedure known. These are listed in table 5 and should be considered tentative procedures, to be modified where necessary with special products (cf. also 99, 100, 101 for further discussion of this problem).

E. *Setting up the assay.* This is merely a matter of mechanical manipulation and need not be further discussed. With large assays, it may be a time-consuming operation; and with highly light-labile vitamins (riboflavin and vitamin B_6), should be conducted, together with step (D) above, in subdued light or with low actinic glassware. Cannon (139) has described an automatic dispenser which is very useful in decreasing the amount of time required for pipettings in large or routinely conducted assays.

F. *Sterilization of assay tubes and media.* The necessity for maintaining pure cultures of the test organism being employed has been mentioned previously, and it is for this purpose that sterilization of test media is required. This has always been effected by autoclaving. The amount of autoclaving, and the extent of other precautions required to prevent contamination, vary widely from one type of procedure to another. In a yeast method for biotin (52), for example, sterilization was

TABLE 5. TENTATIVELY RECOMMENDED EXTRACTION PROCEDURES FOR VARIOUS VITAMINS

VITAMIN	KNOWN COMBINED FORMS	RECOMMENDED EXTRACTION PROCEDURE AND REFERENCES
Biotin		Variable with product. Hydrolysis with 6N H_2SO_4 at 120° (15 lbs. pressure, autoclave) for 1 to 2 hours appears most generally applicable (17, 53, 127). In some instances, some destruction is reported by this treatment, especially if prolonged (127).
Choline	Phospholipids, acetylcholine	Autoclaving (15–17 lbs.) with 10 cc. of 3N H_2SO_4 or HCl per 100 mg. of dry, ground sample (93, 128).
Folic acid	Pteroyldiglutamylglutamic acid Pteroylhexaglutamylglutamic acid	Autoclave finely divided sample with water, cool, then incubate with a specially prepared enzyme from hog kidney (25) at pH 4.5 for 16 hours at 45°. Other conjugate-splitting enzymes from almond (23) or chicken pancreas (129, 130, 131) have also been used and may give superior results with some samples.
Inositol	Phytic acid, lipositol	Reflux sample with excess 18% HCl for 6 hours. Remove HCl by distillation *in vacuo*. Dissolve residue in water and adjust to appropriate pH (69, 55, 132).
Nicotinic acid	Nicotinamide, coramine, nicotinuric acid, coenzymes I and II	Suspend finely ground sample in sufficient N/1 H_2SO_4 to give estimated nicotinic acid content of 1 μg. per cc. of mixture. Autoclave at 15 lbs. pressure for 15 minutes, cool, neutralize with NaOH (32, 29, 133).
Panthothenic acid	Coenzyme A	Autoclave finely ground sample at pH 6.8–7.0 for 15 minutes at 15 lbs. pressure with sufficient water to give 3–10 μg. of vitamin per 50 cc. Cool, add "Mylase P" in an amount equal to $\frac{1}{10}$ the estimated dry weight of sample, and 2 cc. of 2.5 M sodium acetate. Adjust pH to 4.8 with HCl, incubate under toluene for 12–24 hours at 50°. Dilute to 100 cc., filter, repeatedly if necessary, until a clear filtrate is obtained (134).
Riboflavin	Flavin monophosphate, flavin adenine dinucleotide	The finely divided sample is suspended in not less than 10 times its weight of N/10 HCl, autoclaved 15–20 minutes at 15 lbs. pressure, cooled, adjusted to pH 4.5 by addition of 2.5 M sodium acetate solution, and filtered to remove interfering fatty acids (102, 122). An enzymatic digestion procedure (135) was found (135, 136) also to give excellent results (cf. discussion in 101).

TABLE 5—*Continued*

VITAMIN	KNOWN COMBINED FORMS	RECOMMEND EDEXTRACTION PROCEDURE AND REFERENCES
Thiamine	Thiamine pyrophosphate	The finely divided material is suspended in at least 15 times its weight of 0.1 N H_2SO_4 and digested on a steam bath for 30 minutes. If the pH becomes greater than 1.5, additional acid is added. After cooling, sodium acetate solution is added to a pH of 4–4.5. For each 10 μg. of estimated thiamine, 1 cc. of a 10% solution of taka diastase, clarase, mylase P, or other similar enzyme is added. The mixture is incubated at 45–50° for 3 hours, or overnight (under toluene) at 37° (137). A simpler, enzymatic digestion procedure with papain and clarase (135) has also proved effective (46).
Vitamin B_6	Pyridoxal phosphate Pyridoxamine phosphate	A finely divided sample estimated to contain about 2 μg. of vitamin B_6 is suspended in 180 cc. of 0.055 N HCl. The mixture is autoclaved at 20 lbs. pressure for 5 hours, cooled, and adjusted to proper pH for assay (138).
p-Aminobenzoic acid	Pteroyl glutamic acid and higher conjugates p-Aminobenzoylpolyglutamylglutamic acid	No recommendation possible. Procedures may break down variable amounts of folic acid, etc. cf. (50, 51, 91, 92, 101).

effected by merely steaming at 100°C. for five minutes, and inoculated assay vessels were incubated without covers for the incubation period of 16 hours. No trouble from contamination was encountered, perhaps because the simple medium employed, the low initial pH of the medium, the short incubation period and the heavy inoculation with the test organism made contamination of a sufficient magnitude to be observed highly improbable. When complex media are used, suitable for growth of many organisms, together with long incubation periods and small inocula, the need for aseptic precautions becomes correspondingly greater. For such assays, it is customary to autoclave at 15 pounds pressure for from 5 to 15 minutes.

It should be recognized that during the autoclaving process, deep-seated chemical changes can and do take place in the medium, only some of which have been delineated. Many lactic acid bacteria fail to grow in unautoclaved media (119, 140, 141, 23, 142) and the nature of the substances produced which permit growth is imperfectly understood. In some cases, (e.g., for *Streptococcus faecalis*, 23, 140), these appear to be reducing substances, replaceable by thioglycollate or cysteine. In other cases (e.g., *Streptococcus salivarius*, 142), addition of compounds such as pyruvate or acetaldehyde permits growth in unautoclaved media. In all cases investigated, the effective substances appear to be derived from partial breakdown of the carbohydrate of the medium during autoclaving. Such decomposition of sugars during heating has long been known to occur, especially at neutrality or above, and in the presence of phosphates and oxygen (143). Moderate autoclaving can also render a medium

incapable of supporting growth. Thus, autoclaving a medium of conventional composition (48) for as long as 10 minutes rendered it unsuitable for growth of *Streptococcus faecalis*, apparently due to destruction of cystine (49), an essential amino acid for this organism, through interaction with glucose. Again, the interaction of glucose and amino acids at high temperatures is to be expected from their chemical constitution and has been recognized to occur for some time, (cf. 144).

Other recognized effects of autoclaving with the medium which may disturb certain microbiological assays include *a*) the production from pyridoxine, itself inactive in promoting growth of lactic acid bacteria, of small amounts of pyridoxamine and pyridoxal (48), *b*) the conversion of pyridoxal, by transamination reactions, into pyridoxamine (145), *c*) the destruction of thiamine which can be prevented, however, by appropriately modifying conditions of sterilization (46).

These results indicate that more attention needs to be given to the possible disturbing effects of variable autoclaving in microbiological assays, for both vitamins and amino acids. Ordinarily, any minor effect of autoclaving would escape notice because both standards and sample would be equally subject to it. Positional differences in an autoclave, however, could result in different tubes receiving different heat treatments and thus contribute to variability in an assay.

G. *Inoculation with the test organism.* Most commonly, inoculum is added dropwise from a pipette to each assay tube. In most cases, the final amount of growth obtained is dependent almost solely on the concentration of limiting vitamin present and is not materially influenced by fluctuations in drop size. In some cases (e.g., in biotin assay with *L. casei*, 20), inadvertent variation in the size of inoculum will materially affect the result, especially if a heavy inoculum is used. Such variation was successfully eliminated either by reducing the amount of the inoculum (by diluting the inoculum suspension) or by carefully standardizing the amount added. A syringe arrangement said to be useful for the latter purpose has been described by Black and Arnold (146). Another useful device for insuring equal inocula for different assay receptacles is to sterilize the basal medium separately, before its addition to the assay tubes, cool, inoculate, then dispense aseptically to the sterile assay vessels. This procedure has been widely and successfully used in assays with yeast (cf. 52, 53). Alternatively, the inoculum used can be diluted, and a comparatively large volume (e.g., 1 cc.) used to inoculate each tube.

H. *Incubation.* The effect of variations in temperature of incubation on assay results and the manner in which length of incubation is related to the specificity of an assay have been considered previously. For accurate assay, it is essential that all tubes be incubated at the same temperature. Small cabinet-type incubators do not generally maintain sufficiently uniform temperatures throughout the cabinet to permit the best results (cf. 147). Circulating air incubators or well-stirred water baths are much superior. It has also been found (148) that where water baths are used, care should be taken that circulating water does not impart variable vibration to the assay tubes, since variations in growth response may result.

I. *Determination of response to the vitamin.* The response of lactic acid bacteria to additions of the test substance is most commonly followed by titrating the acid produced during growth. In complete media containing one per cent of glucose, many

of these organisms (e.g., *Lactobacillus arabinosus*, *Lactobacillus casei*) will convert this almost quantitatively to lactic acid, producing from 10 to 11 cc. of 0.1 N acid per 10 cc. of medium. Larger quantities of acid (17 to 20 cc.) will be produced if two per cent of glucose is added and the buffer capacity of the medium is sufficiently high. Less acid-tolerant organisms (e.g., *Streptococcus faecalis*, *Leuconostoc mesenteroides*) will produce as much as 12 to 15 cc. of 0.1 N acid under similar conditions. This range is a very convenient one for titration. By using microburettes, with more dilute alkali for titration, the assays can readily be adapted to a 'micro' scale, in which the total volume of medium is 0.2 cc. or even smaller (149, 150), and such procedures have proved useful for some purposes.

Despite the fact that turbidimetric assays have been less widely made, comparative assays (117, 21, 151, 152, 153) indicate that this procedure gives results as reliable as those obtained by titration. When turbidity measurements are made samples assayed must be optically clear, and turbidity not due to cells must not develop on incubation. The organisms must be uniformly suspended by shaking before measurement. A moment or two is allowed after shaking to permit any air bubbles to rise and break. Any stable photoelectric colorimeter may be used. By proper choice of the wave length at which measurements are taken, interference from the slight color imparted to the medium by some samples is usually readily avoided. The procedure is somewhat faster than titration.

Turbidity measurements are used exclusively in yeast growth assays (table 2) and for almost all assays using miscellaneous bacteria (table 3). In no case has difficulty been reported to arise from this method of measurement, except where extremely dark-colored samples were assayed.

In assays with *Neurospora* and other filamentous fungi, the mycelium is harvested with a needle or filtered from the medium, washed, dried and weighed. Although more laborious than titration or turbidity measurements, accuracy of assays conducted in this manner is very satisfactory.

In all cases, it is possible to make the comparison of responses to standard and sample either before or after growth has stopped for lack of the essential vitamin. In the former case, one measures comparative growth rates; in the latter, the extent of growth. Reasons for believing that the latter procedure is the preferable of the two (with any one organism) have been considered earlier.

J. *Calculation of the results.* The response to various concentrations of the standard vitamin is plotted against the concentrations to produce a *standard curve.* Most commonly, the amount of the vitamin present in any given sample tube is determined by interpolation onto this standard curve. From simple dilution factors the value for the original sample is readily calculated. If properly set up, several increasing concentrations of the sample yield responses which fall in the assay range of the standard curve. Values for the vitamin content of the sample calculated from each of these concentrations should check within the limits of error of the assay (usually considered to be about ± 10 per cent for the better microbiological assays). If they do so, the values are averaged to obtain the final result. Sometimes it is found that the assay values calculated from several such levels of the sample will show a steady rise (or fall) with increasing concentration. Such a 'drift' in values indicates that

the test organism is not responding to the sample in the same way it does to the pure standard and is evidence against the validity of the assay. Some known reasons for such 'drifts' will be considered below.

Recently, a number of investigators (154-157) have attempted to devise mathematically sound procedures to supplement, and in some cases replace, the empirical method described above for calculating results of assays. Where the dose-response curve to the test substance is a straight line, or where it can be converted into a straight line by a suitable transformation of response, such procedures are already available (155, 157). When combined with a statistically valid design for setting up the assay, they permit use of the microbiological data themselves to determine the variance and standard error of the assay result. For this reason they promise to be most valuable for some purposes. This subject has been fully discussed by Bliss (158). Application of certain findings made in connection with these studies may change the mechanics of assay considerably. Customarily, for example, each concentration level of standard and sample is prepared in duplicate, and increasing concentration levels are prepared, inoculated, incubated and the response measured in consecutive, nonrandom order. Such a procedure is statistically unsound, and investigation has shown (159) that under these conditions, excessively good agreement, which does not reflect the true degree of variation encountered in the assay as a whole, is obtained between duplicate tubes. Replicate points prepared in this manner, therefore, may be largely valueless. If, however, duplicate tubes are prepared, inoculated, etc., in random order, agreement between them is not so good, and their variance is a true reflection of the precision of the method. Similarly, current practice usually calls for several more points for the standard curve than for the sample curve. Where the standard curve is regular and its nature well established, however, this procedure is valueless, for the final estimate of potency cannot be more accurate than the least accurate observation made in obtaining it. Logically, the number of points determined for the standard need be no more than that determined for each sample.

CRITERIA FOR ESTABLISHING RELIABILITY IN AN ASSAY. The general procedures available for establishing whether or not an assay method is yielding reliable results have been fully discussed elsewhere (133, 160) and need only be listed here. They include *a*) agreement with results of other reliable methods, *b*) obtention of theoretical recoveries on addition of known amounts of vitamin to a sample, *c*) obtention of consistent values on repeated assay, *d*) agreement in assay values obtained with different assay organisms having different nutritive requirements, and *e*) agreement of results calculated from different assay levels, and the absence of 'drift.'

Unfortunately, (*a*) is applicable in only a few instances, since the reliability of other methods for determination of a vitamin is usually established no better than that of proposed microbiological methods. If methods as widely different as chemical and microbiological assays do give the same values on a given extract, it is, however, strong presumptive evidence for the validity of both procedures; (*b*) and (*c*) are obvious requirements of any analytical method; although nonfulfillment is certainly sufficient to condemn an assay, neither good recoveries nor consistent values on repeated assay suffice to prove a method reliable. Points (*d*) and (*e*) are more con-

clusive. If different assay organisms having different nutritive requirements give values which check, it is excellent evidence for the reliability of the values, since it is improbable that the different organisms would respond to interfering materials in exactly the same manner and to exactly the same extent. The absence of 'drift' in assays is evidence that the assay organism is responding to the vitamin and the sample in exactly the same way and is thus excellent evidence for the absence of interfering materials in the sample, and hence for specificity of the assay.

'DRIFT' AND ITS POSSIBLE CAUSES. It has been emphasized above that occurrence of a regular shift or 'drift' in assay values, either increasing or decreasing with concentration of the sample, is evidence for the presence of interfering materials in the sample, and that where such drift occurs, complete reliance cannot be placed on the assay result. Such drifts are quite frequently found in assay work, and consideration of some cases in which their cause is known may suggest procedures for eliminating them in other cases as well.

In several instances 'drifts' in assay values can be produced by assay of a known substance or of mixtures of known substances. Three general cases have been recognized, as follows.

(A) A substance chemically unrelated to the vitamin may stimulate or inhibit response of the test organism to suboptimal levels of the vitamin. The effect of free fatty acids on the response of *L. arabinosus* and *L. casei* to pantothenic acid, and of *L. casei* to riboflavin, is an example (122–125, 44). Depending upon the concentration, the fatty acid may stimulate or inhibit the growth response to riboflavin. At levels ordinarily encountered in samples, stimulation occurs, but the per cent stimulation varies with the amount and type of fatty acid, so that a drift in assay values is obtained at increasing levels of sample. In both the riboflavin and pantothenic acid assays, this effect is eliminated by filtration of the sample at pH 4.5 (122), which removes the interfering lipids. In the latter assay, it can also be avoided by addition of an excess of oleic acid to the basal medium (40).

(B) Substances chemically related to the vitamin may replace the vitamin in the nutrition of the test organism, but the dose-response curve to the vitamin and the related compound may be entirely dissimilar. In such a case, the ratio of growth-promoting activity of analogue to that of vitamin is not constant at various concentration levels, and if both substances are present in a sample, drift results. For example, Bird and Robbins (161) showed that the activity for *L. casei* of partial enzymatic hydrolysates of vitamin B_c conjugate (pteroylhexaglutamylglutamic acid) in terms of free vitamin B_c (folic acid, pteroylglutamic acid) varied greatly, increasing as the dosage level was increased. This conjugate occurs naturally, and when incompletely hydrolyzed samples are assayed with *L. casei* against a standard of pteroylglutamic acid, upward drift at increasing levels of sample is encountered. A similar example is provided by work of Rabinowitz and Snell (162), who found that pyridoxamine phosphate, which occurs naturally, was active in place of pyridoxamine for *Streptococcus faecalis*. Its activity varied from 0.5 to 2.0 times that of pyridoxamine when tested at various levels. Incompletely hydrolyzed samples which contained this substance showed marked drift when assayed. In both of the above instances, drift could be eliminated by appropriate treatment of the sample to completely hydrolyze active combined forms of the vitamin to the free compounds.

(C) Substances physiologically related to the vitamin, in that they may replace it for growth, may occur naturally, and interfere in the same way as described under (B) above. Thymine, for example, replaces folic acid under the testing conditions used with lactic acid bacteria, and if present in sufficient quantity in natural extracts, will interfere with assays for folic acid (163). In a similar fashion, oleic or linoleic acids replace biotin for lactic acid bacteria so far suggested for its assay (164, 165, 166), and D-alanine may replace vitamin B_6 (167). Where interferingly large amounts of these substances were present in samples for assay, the only remedy would be adoption of an extraction procedure which would eliminate them from the sample, adoption of a different test organism unaffected by the interfering material or development of assay conditions under which interference no longer occurred.

SPECIFICITY OF TEST ORGANISMS. A mistaken impression of the specificity of most test organisms might be gained from examination of the instances in (C) above. As a general rule, microorganisms show marked specificity in their requirement for individual vitamins. For example, animals can satisfy their nicotinic acid requirements from dietary nicotinic acid or nicotinamide, or by synthesis from tryptophane (168, 169). Microorganisms so far used for assay are unable to utilize tryptophane in place of nicotinic acid. While the most widely useful assay organisms utilize nicotinamide, nicotinic acid and coenzymes I and II with equal facility (as do animals), other microorganisms are available which require either free nicotinic acid, nicotinamide or the intact coenzymes I and II (133). Similarly for thiamine, microorganisms are known which require only the thiazole portion of the molecule, only the pyrimidine portion, both thiazole and pyrimidine or intact thiamine (170). By using such microorganisms as assay agents, useful information concerning the metabolism of vitamins can be obtained (e.g., 171, 172). Such procedures lie outside the scope of this review and have not been listed in tables 1 to 3. Knowledge of the specificity of response of an organism to a vitamin and its naturally occurring derivatives or degradation products is very helpful in devising valid procedures for assay. The pertinent information so far available for most widely used assay organisms has been summarized elsewhere (99, 101) and will not be further considered here.

PREFERRED METHODS OF ASSAY. The number of publications dealing to some extent with the problem of microbiological assay of vitamins and the number of alternative methods for a single vitamin have become so large that it is useful to list what may be called 'preferred' methods of assay. These are given, together with certain substances which may interfere in their accurate use, in table 6. Any such list of methods will vary to some extent with the experience and personal preferences of its author. The tentative and temporary nature of any such list should also be clearly apparent. In compiling the present list the following points have been given most emphasis:

a) Widely tested procedures which have proved generally satisfactory are preferred over others which, though they may possibly be superior, have not been tested by widespread use.

b) Since the most widely used assay procedures employ lactic acid bacteria, and since all methods which employ these organisms are very similar, assay methods using these organisms are preferred in the absence of clearly superior methods.

c) From the standpoint of simplicity and speed, assays employing yeast are usually preferable to those using lactic acid bacteria, and these in turn to those using *Neurospora.*

In each case, the method listed should be used with the most suitable extraction procedure known (table 5), which may or may not be that described with the method. In some cases, where there appears little choice between two or more methods on the basis of the above criteria, more than a single method is given.

TABLE 6. PREFERRED METHODS FOR MICROBIOLOGICAL DETERMINATION OF THE VITAMINS

VITAMIN	TEST ORGANISM	REFERENCE	KNOWN INTERFERING MATERIALS
p-Aminobenzoic acid	*Neurospora crassa* (mutant)	(73)	
Biotin	*Lactobacillus arabinosus*	(17)	Oleic and linoleic acids
	Saccharomyces cerevisiae	(53, 54)	Desthiobiotin
Choline	*Neurospora crassa* (mutant)	(73, 93)	Methionine
Folic acid	*Lactobacillus casei*	(21, 26)	Folic acid conjugates, thymine
	Streptococcus faecalis	(26)	Thymine
Inositol	*Saccharomyces carlsbergensis*	(55, 58, 67)	
Nicotinic acid	*Lactobacillus arabinosus*	(16, 29, 32)	
Pantothenic acid	*Lactobacillus arabinosus*	(40)	Fatty acids
	Saccharomyces carlsbergensis	(58)	
Riboflavin	*Lactobacillus casei*	(42, 137)	Fatty acids
Thiamine	*Lactobacillus fermenti*	(46, 47)	Cocarboxylase
	Saccharomyces cerevisiae	(60)	
Vitamin B_6			
Total (pyridoxal + pyridoxamine + pyridoxine)	*Saccharomyces carlsbergensis*	(67)	
Pyridoxamine + pyridoxal	*Streptococcus faecalis*	(23)	Pyridoxamine phosphate, D-alanine
Pyridoxal	*Lactobacillus casei*	(49)	D-alanine

CONCLUDING REMARKS

The attempt has been made above to summarize, in general terms but with specific examples, certain basic information helpful in understanding and using microbiological assay methods. No attempt at an exhaustive treatment of the literature has been made. All of these assay procedures are based on the controlled growth of living organisms. In discussing procedures based on such a complex and imperfectly understood phenomenon, it is difficult to avoid making statements which appear logical but which may be based on untested assumptions. Some such assumptions have been discussed in the body of this review. An additional example is the statement, often heard, that microbiological assays cannot be more precise than ±10 per cent because of the 'biological variability' of the test organisms employed. As indicated elsewhere (96, 98), the large numbers of organisms

involved in microbiological assays should free the result of variation attributable to 'biological variation,' and actual experimental results conducted with pure vitamins (150) indicate that much more precise results than this can be obtained under favorable circumstances. Present procedures are rarely more precise than this when applied to natural materials, but this should be ascribed to our improper understanding and control of variables in the assay procedure, and not to the assay organisms. The future improvement of these methods would appear to lie, then, in an improved understanding of processes involved in growth and metabolism of the test organisms, which would permit more precise control of those factors at present leading to variation.

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